

The immunochemistry of the cell surface antigens of  
*Pasteurella haemolytica*

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DECLARATION

The work reported in this thesis was part of a larger project concerned with the investigation of pasteurellosis in sheep, and consequently some of the experimental findings were obtained in collaboration with my colleagues at the Moredun Research Institute. The work carried out on this thesis was carried out by myself, except for some areas of collaborative experiments which were clearly indicated. A full role was played in the design of the experiments and the interpretation of the results.

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## ABSTRACT OF THESIS (Regulation 79)

Name of Candidate ..... William Donachie  
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The aim of this study was to identify, isolate and characterise the protective antigens present on the surface of Pasteurella haemolytica, serotype A1. A specific serotype antigen was identified by crossed immunoelectrophoresis (CIE) in a hot phenol-water extract (PWE) of P. haemolytica A1 cells. This preparation protected mice challenged with the homologous serotype. However, this protection and also the serotype-specific antigenicity of the PWE was reduced by treatment with sodium periodate but not proteinase K or heat, indicating that the protective antigen was carbohydrate in nature. The composition of PWE consisted of carbohydrate with some protein and lipid. The sugar analyses revealed the presence of mannose, galactose, glucose, two heptoses, glucosamine and galactosamine. The additional presence of ketodeoxyoctonic acid suggested that lipopolysaccharide was the major component of PWE. Partial acid hydrolysis and subsequent separation of the products on Sephadex G50 indicated that the LPS may be of the rough type. However, two antigens which were identified in the PWE by CIE could not be separated from each other to allow individual analyses of both.

The PWE and sodium salicylate extracts (SSE) antigens of P. haemolytica A2 and A6 were also investigated. The PWE of both serotypes were found to have similar compositions to that of A1 PWE, especially the sugar composition determined by gas chromatography GC where identical sugars were present, although in different proportions. A strong serotype-specific antigen was observed in A6 PWE when analysed in CIE but the reaction with A2 PWE was weak. The PWE of A6 protected against homologous and a heterologous A1 challenge indicating some sharing of antigens within the carbohydrate component of A1 and A6 but no consistent protection was observed with any inactivated A2 vaccine although a combination of concentrated SSE and heat killed organisms did show protection on one occasion. Solid protection was observed in mice which had survived an LD<sub>50</sub> dose of A2 and had then been rechallenged.

Strains of P. haemolytica not typable by the indirect haemagglutination test (IHA) isolated from both sheep and cattle were investigated using the immunological methods applied to A1. These strains were shown to belong to the A biotype and were groupable on the basis of specific antigenicity in agarose gel diffusion and CIE. A counter current immunoelectrophoresis (CCIE) method was developed to detect these antigens. Ten serogroups were identified in 31 strains by CCIE and these groups contained both cattle and sheep strains. Protection was evident in mice when SSE antigens of these strains were used as vaccines but a high level of cross protection was evident between IHA negative serogroups. Representative strains of 2 IHA negative serogroups, shown to be of high and low virulence for mice, were not found to be pathogenic for sheep.

A 'sandwich' enzyme-linked immunosorbent assay (ELISA) using rabbit IgG as an antigen trapping layer was developed to measure antibody in the sera of sheep and mice to serotype-specific antigens of P. haemolytica.

A modified ELISA test and the IHA test were used to follow the development of serum antibodies in sheep with an experimental, chronic pasteurellosis. There was a specific serum antibody response to P. haemolytica A2.



ABBREVIATIONS

AGD	-	Agarose gel diffusion
CCIE	-	Countercurrent immunoelectrophoresis
CFA	-	Complete Freund's adjuvant
CIE	-	Crossed immunoelectrophoresis
EDTA	-	Ethylene diamine tetra acetic acid
ELISA	-	Enzyme-linked immunosorbent assay
EM	-	Electron microscopy
FRIE	-	Fused rocket immunoelectrophoresis
GC	-	Gas chromatography
HE	-	Heat extract of <u>P.haemolytica</u> cells
IFA	-	Incomplete Freund's adjuvant
IgG	-	Immunoglobulin G.
i.p.	-	Intraperitoneal
i.v.	-	Intravenous
LPS	-	Lipopolysaccharide
MRI	-	Moredun Research Institute
PI <sub>3</sub>	-	Para influenza virus type 3.
PWE	-	Phenol-water extract
SBA	-	Sheep blood agar
SDS	-	Sodium dodecyl sulphate
SDS page	-	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPF	-	Specific pathogen free
SSE	-	Sodium salicylate extract
RBC	-	Red blood cell
wpi	-	Weeks post infection

## GENERAL INTRODUCTION

Pneumonia in sheep is the cause of major loss to the sheep farming industry of Great Britain and an idea of its importance is given in a survey conducted by the Ministry of Agriculture, Fisheries and Food (MAFF 1964) in which it was found that 82% of all carcasses referred to Veterinary Investigation Centres showed evidence of pneumonia. There is no reason to believe that the situation has changed much since then and a Veterinary Investigation Diagnosis analysis report for the years 1976-77 showed that pasteurellosis is the pneumonia found most frequently in sheep. The organisms implicated in the disease are Pasteurella haemolytica and Pasteurella multocida with the former being by far the more significant (Gilmour, 1978a).

Evidence by various investigators has indicated that Pasteurella species are also the main causative organisms of pneumonia in sheep throughout the world. Hamdy et al (1959). found that pneumonia in slaughtered lamb was associated with P.multocida in 15 out of 51 cases and P.haemolytica in 9 out of 51 cases. In an investigation of enzootic pneumonia in South Africa, Van der Veen and Zumpt (1967) recovered P.haemolytica from 13 out of 36 outbreaks and P.multocida from six. A 3 year study of perinatal lambs mortality in Western Australia by Dennis (1974) indicated that P.haemolytica was the main cause of pneumonia. Alley (1975) in a study of 5-10 month old lambs at a New Zealand slaughterhouse isolated P.haemolytica in pure culture from 45% of all cases and in association with Branhamella cattarhalis from a further 14%.

In all these studies a number of other bacteria were found

including Escherichia coli, Staphylococcus species, Streptococcus species, Pseudomonas species, and Fusobacterium necrophorum. These were thought to be of minor importance although Stevenson (1974), working in Canada, reported cases of pneumonia in sheep due to infection with Streptococcus zooepidemicus which he was able to reproduce experimentally.

Pneumonic pasteurellosis is not restricted to sheep but is also a major problem in the cattle industry. Losses in the U.S.A. due to "shipping fever", as the disease is sometimes known in North America, were estimated by McKercher (1978) at 76 million dollars in 1972. The involvement of P.haemolytica in the cattle disease has been well documented and excellent reviews are available in the literature (Yates, 1982; Carter, 1967; Gilmour, 1978b).

This thesis concentrates on P.haemolytica as the chief cause of ovine pneumonic pasteurellosis.

Control of the disease is likely to be best achieved by the use of vaccines against P.haemolytica and recent work by Gilmour et al (1979) has shown that vaccines prepared from extracts of bacterial cells do protect sheep against experimentally produced pneumonic pasteurellosis. A more detailed knowledge of the important antigens involved in stimulating protection could lead to improvement in the production and efficiency of existing vaccines. The aim of this thesis was to attempt the isolation and characterisation of the surface antigens of P.haemolytica.

The first part of the literature review discusses the literature associated with P.haemolytica and pneumonic pasteurellosis, the second part is concerned with the structure

and chemistry of the Gram-negative cell wall and the final part with the use of bacterial surface antigens as vaccine components.

Pasteurella haemolytica and pneumonic pasteurellosisCharacteristics of Pasteurella haemolytica

P.haemolytica has been the subject of intense investigation since Jones (1921) first described a short Gram-negative, non motile, capsulate cocco-bacillus isolated in a slaughterhouse survey. In 1932 Newsom and Cross isolated a similar organism from a pneumonic sheep lung and gave it the name we now associate with the bacterium.

P.haemolytica is a small Gram-negative pleomorphic rod showing marked bipolar staining when freshly isolated from animal tissue. It is aerobic and facultatively anaerobic, oxidase and catalase positive and ferments sugars by fermentation, giving small amounts of acid but no gas.

It can be easily distinguished from other members of the genus Pasteurella and especially P.multocida, by the characteristics shown in Table 1.

Strains of P.haemolytica can be classified into two biotypes and at least fifteen serotypes. This aspect of P.haemolytica has been the subject of reviews by Biberstein (1978) and Gilmour (1978a;1980).

Biotype: Smith (1959b;1961) investigated the biochemical and cultural characteristics of isolates of P.haemolytica. This resulted in the division of P.haemolytica into two biotypes, A and T, which denoted arabinose fermentation and trehalose fermentation respectively. Smith also showed that the biotypes differed in their sensitivity to penicillin, with biotype A



Table 1

Differentiation of P.haemolytica from other Pasteurella species.

- 
1. P.haemolytica causes weak haemolysis on 7% sheep blood agar plates. A layered plate with a nutrient agar underlayer and blood agar overlayer facilitates the observation of this haemolysis, P.multocida is not haemolytic.
  2. P.haemolytica does not produce indole whereas P.multocida does.
  3. P.haemolytica will grow on MacConkey agar while P.multocida will not.
  4. P.haemolytica does not produce urease while P.ureae and P.pneumotropica do.
-

being more susceptible. This has been confirmed in subsequent studies by Biberstein and Kirkham (1979), who showed that biotype A strains also had increased in vitro susceptibility to ampicillin, cephalothin, chloramphenicol, tetracycline, erythromycin and nitrofurantoin. Olmos and Biberstein (1979) showed that there were differences in the resistance to dyes apparent between the biotypes. T strains grew in brain heart infusion broth containing basic fuchsin ( $0.2 \text{ ug ml}^{-1}$ ) while A strains did not.

Taxonomic studies by Smith and Thal (1965) on the A and T biotypes led them to propose that they could be considered as different species and the differences noted in nucleic acid homologies (Biberstein and Francis, 1968) and in polypeptide content as measured on polyacrylamide gels (Thompson and Mould, 1975) gives support to this proposal.

Serotype: Biberstein et al (1960) developed an indirect haemagglutination (IHA) test to serotype strains of P.haemolytica and identified 10 serotypes. In 1962, an investigation into the relationship of A and T biotypes to the serotypes (Biberstein and Gills, 1962) disclosed, in the sample studied, a consistent association between serotypes and biotypes. Subsequently, five additional serotypes, have been added (Biberstein and Gills, 1962; Biberstein and Thompson, 1966; Pegram, 1979; Fraser et al, 1982b) so that at present 15 serotypes are recognised. Biotype A comprises 1,2,5,6,7,8,9,11,12,13 and 14 and Biotype T 3,4,10 and 15. The serotype antigen is presumed to be a surface antigen which is either polysaccharide (Cameron 1966) or lipopolysaccharide in

nature (Carter 1967). A number of strains which are untypable by the IHA test have been isolated from both healthy and diseased sheep. These generally belong to the A biotype and their negative reaction in the IHA test has been ascribed by Aarsleff et al (1970) to a lack of the soluble antigen responsible for serotyping.

Strains of P.haemolytica can also be serotyped by the rapid plate agglutination method (RPA) described by Frank and Wessman (1978) where direct agglutination of the bacterial cells can be carried out on a microscope slide. Recently Frank (1980) tested 10 untypable strains using this method and discovered that they fell into three distinct serogroups. He proposed that that these untypable strains lack the necessary soluble antigen for the RPA. He did not feel that the existence of these serogroups undermined the theory put forward by Aarsleff et al (1970) that untypable strains were traditional serotypes lacking the soluble surface antigen.

The distribution of biotypes and serotypes is of epidemiological interest and surveys carried out by Thompson et al (1977) and Fraser et al (1982a) on P.haemolytica strains isolated from field cases showed that during the years 1974-77 A biotypes comprised 52% of all strains serotyped while between 1978-81 56.5% of all strains were A biotypes. In both surveys A2 was the serotype most frequently isolated; 33% of all serotypes in 1974-77 and 38% in 1978-81. T biotypes were isolated from 42% of all cases in 1974-77 and from 33.5% in 1981. However the T distribution was complicated by the discovery of T15 midway through the timespan of the later

survey. Fraser and his colleagues also noted that over the period of the two surveys the rise in frequency of T15 from 0 to 30% of T strains tested coincided with a dramatic drop in the incidence of T3 from 38.5% to 9.4%. They suggested that some strains previously identified as T3 were in fact T15 as the strains possess a common antigen which leads to a cross reaction in the IHA test.

A13 and A14 were not included in the second survey but were isolated from a few cases, mainly exotic animals. The extension of the serotype range did not reduce the frequency of untypable strains and these strains did in fact increase in frequency from 6-11%. Gilmour (1980) suggests that the untypable group may harbour more type strains which have not as yet been detected.

#### Pasteurellosis in sheep

Smith (1961) showed that the disease in sheep caused by P.haemolytica fell into two distinct syndromes each of which is generally associated with one of the two biotypes.

Disease caused by biotype A: Montgomerie et al (1938) described this form of disease as an enzootic pneumonia and it has since been shown to affect sheep of all ages. However, in lambs under two months old a more systemic form may occur (Gilmour, 1980). Morbidity and mortality can be upto 10% of the flock but sheep which recover can remain chronically unthrifty. Stress factors would seem to play an important part in the disease and such factors as temperature , dipping, castrating and dosing for treatment of helminthiasis have all been

postulated as predisposing the animals to disease (Gilmour 1978).

Viral infection prior to pasteurellosis has been put forward as a possible predisposing factor and such viruses as para influenzae virus type 3 (PI3) and respiratory syncytial virus (RSV) have been implicated. Serological and experimental evidence obtained by Davies et al (1981) in New Zealand and Sharp et al (1978) in the U.K. lend weight to this argument.

Gilmour and Brotherston (1963) first described a sub-acute form of the disease in which there is now known to be a close association of P.haemolytica biotype A strains with Mycoplasma ovipneumoniae in lung lesions. Jones et al (1979) have shown that sub-acute disease can be reproduced by the inoculation of homogenated lung lesions from affected animals which have been shown to contain only P.haemolytica and M.ovipneumoniae.

Bacteriological confirmation of A type pasteurellosis is usually obtained when an A biotype strain is recovered, generally in pure culture, from exudates or lung lesions in numbers approaching  $1 \times 10^7$  colony forming units (cfu) per g of lung. Recovery of P.haemolytica from other organs such as the liver, spleen, kidney, lymph nodes or heart blood is possible when young lambs or very acute cases are examined. Gilmour et al (1982) have shown that in the sub acute form of the disease P.haemolytica can be recovered from the lesions up to six weeks after infection.

Disease caused by biotype T: A rapidly fatal septicaemic disease in lambs, associated with P.haemolytica was first described by Stamp et al (1955) in Scotland and then later by

Biberstein and Kennedy (1959) in the United States. Smith (1959b) subsequently identified the strains involved in the disease described by Stamp as T biotypes. Dyson et al (1981) suggested that this disease be termed a systemic disease as it was doubtful that the bacteria underwent a true septicaemic phase with rapid multiplication in the blood.

This form of the disease affects lambs 5-12 months old, usually in the autumn and is coincidental with husbandry changes such as movement to improved pasture or feeding on rape and turnips. Although mortality from this disease is generally low it may reach 20% of the sheep at risk.

P.haemolytica T biotype strains are present in all body organs in large numbers and in pure culture if death has been fairly recent (Gilmour, 1978). The T biotype disease has a complex aetiology and will not form any part of this thesis which will concern itself solely with A biotype strains.

Control of the disease: Although P.haemolytica is readily susceptible to antibiotics (Thompson, 1973) it is generally accepted that vaccination probably offers the most effective means of control.

One of the earliest reports of successful vaccination was that of Dungal (1931) in Iceland, where he described the vaccination of sheep with either 1ml of a live 12 hour broth culture or 1ml of a phenolised 12 hour culture given subcutaneously. Although this protected 66 out of 70 sheep against subsequent challenge with the homologous strain the work was never repeated successfully.

In 1957 Stevens described the vaccination of sheep with a

formalinised autologous culture given subcutaneously. This treatment was effective in stopping losses in 14 outbreaks. Although few controls were included the results were thought to be encouraging.

Cameron (1966) claimed to have demonstrated successful immunization of sheep by vaccinating with whole cells, polysaccharide or proteins of P.haemolytica and showing passive protection of mice given serum from the vaccinated sheep. However, he states in a subsequent report (1970) that it was impossible to infect laboratory animals consistently and effectively which casts doubt on the earlier report.

After using an autogenous killed cell vaccine, Van der Veen and Zumpt (1967) reported that losses in the flock given the vaccine were reduced from 15-20% to 1-2%.



### Experimental reproduction of pneumonic pasteurellosis

One of the major difficulties confronting workers investigating pasteurellosis was the successful experimental reproduction of the disease. P.haemolytica is ubiquitous in its relationship with sheep and isolates can be recovered from the respiratory tract of a high proportion of normal sheep. Gilmour et al (1974) in a study into the prevalence of P.haemolytica in sheep tonsils reported that 95% of all tonsils and 64% of all nasopharynxes examined contained P.haemolytica. The A and T biotypes differed in their distribution between these two sites with 65% of all isolates from tonsils and 6% of isolates from the nasopharynx being T biotype strains. This was in general agreement with an earlier study by Biberstein and Thompson (1966) who reported that 85.4% of all P.haemolytica isolates recovered from the nasopharynx of normal sheep were of the A biotype while only 2.2% were of the T biotype. In both these studies a random assortment of serotypes was encountered. Studies by Al Sultan (1982) have shown that colonization of the respiratory tract occurs quickly after birth.

Dungworth and Cordy (1962) using chlamydiae alone in an intra-tracheal challenge found P.haemolytica in the lung lesion indicating the opportunistic nature of the bacterium. Biberstein et al (1967) challenged sheep with P.haemolytica by an intubation method. This resulted in lesions similar to natural field cases but did not cause death. When combined with chlamydiae increased numbers of animals had lesions suggesting synergy in the aetiology of the disease. An unsuccessful attempt to produce experimental pneumonia using PI3 and



P.haemolytica A5 in combination led Biberstein to conclude that PI3 was not significantly involved in the disease.

Gilmour and co-workers (1975) produced pneumonic pasteurellosis identical to that seen under natural conditions by exposing specific pathogen free (SPF) lambs to an aerosol of P.haemolytica serotype A1. This resulted in 40% of the lambs developing pasteurellosis. Subsequent experiments showed that if the bacterial aerosol was preceded one week earlier by an intra-tracheal challenge with PI3 virus the percentage of infected animals rose to 90% (Sharp et al, 1978).

This system of experimentally produced pasteurellosis allowed the testing of vaccines in a controlled environment and Gilmour et al (1979) reported successful immunization of SPF lambs with extracts of P.haemolytica cells obtained by treatment with sodium salicylate. Protection was stated to be serotype specific as a challenge with a serotype not included in the vaccine resulted in failure of the vaccine to confer protection.

#### Laboratory animal models for the disease

In parallel with the search for an effective experimental method for the reproduction of pasteurellosis in sheep researchers were also investigating possible laboratory animal models. The attraction of the latter lies in less costly experiments and in the availability of greater numbers of animals.

However, P.haemolytica is relatively non-pathogenic for laboratory animals unless high numbers of bacteria are used and thus increase the likelihood of endotoxic shock.

Smith (1959c) described two successful methods for infecting mice with P.haemolytica. One was an intra-cerebral inoculation of the organism suspended in casein hydrolysate solution. The organism multiplied and caused death but there was variation in individual susceptibility to the infection. A modification of this method where inoculation of a starch-saline solution which caused brain trauma, was followed by intra-venous inoculation of P.haemolytica, gave 100% mortality. However this was dependent on a suitable dose of P.haemolytica. The other method described was a challenge by the intra-peritoneal route with a suspension of the bacteria in mucin. This caused death following the development of septicaemia after 48 hours. The number of bacteria required for successful challenge was less than that causing endotoxic shock and the challenge seemed reproducible. Smith used this model to demonstrate passive protection and later active immunization of mice against challenge (Smith 1959a).

This model was applied to the investigation of a role played by "capsular" and somatic antigens in immunity by Biberstein and Thompson (1965). The major influence seemed to reside in the "capsular" antigen with somatic antigens being of minor importance. Knight et al (1969) using the same model could not repeat the work and found anomalies in their results, one of these being that greater immunity was conferred by antigenically unrelated serotypes.

The problem of inconsistent results was encountered by Cameron and Smit, (1970) while trying to reproduce an intraperitoneal (i.p.) challenge with an aqueous suspension of

organisms that he had described earlier (Cameron 1966). He concluded that he was unable to challenge mice consistently using this method.

A different approach was attempted by Rushton (1978) who inoculated mice, under anaesthesia, with  $10^{6.6}$  to  $10^{7.6}$  cfu of P.haemolytica. This caused a non-fatal pneumonia after three days and it was claimed that the model was quick, easy, cheap and "provided an opportunity for studying P.haemolytica in the lung without the development of extensive changes and subsequent death".

In an investigation of mouse models for P.haemolytica Evans (1979) showed that the pneumonia seen by Rushton was due to toxicity and not to multiplication as the same effect could be obtained by the inoculation of killed cells.

Evans and Wells (1979a) described a modification of Smith's original ip model where after challenge with P.haemolytica suspended in mucin, the multiplication of the bacteria was measured by counting the number of bacteria in the liver of the mouse. This method seemed to be more reliable than the simple death or survival criteria used by previous researchers and experiments with sodium salicylate extract (SSE) vaccines of P.haemolytica gave similar results to those found by Gilmour et al (1979) in sheep, except that no protection was offered against P.haemolytica serotype A2 challenge.

Mucin has been used to enhance the virulence of organisms which are weakly or non-pathogenic when injected into mice on their own (Olitzki, 1948). The reasons for this enhancement are not fully understood although some specific effects have been

suggested. One of these is that there is a simple protective role due to the viscosity of the mucin suspension and other work with Neisseria meningitidis has shown that the presence of iron in mucin is an important factor (Calver et al, 1976). Al Sultan (1982) found that when iron is given intravenously some hours before a suspension of P.haemolytica in mucin is given ip then the LD<sub>50</sub> is reduced significantly below that obtained for mucin alone. This indicates the importance of iron in the virulence of P.haemolytica for mice.

The use of SSE of P.haemolytica described by Gilmour et al (1979) was prompted by the observation that existing commercial vaccines were of questionable efficacy in preventing pasteurellosis (Gilmour, 1980) of low potency and limited serotype range and indicated that there was a need for an improved, low dose volume, potent, multi-serotype vaccine against pasteurellosis.

### The Gram-negative cell envelope.

Treatment with sodium salicylate is thought to strip the outer coat from the bacterium as the antigen responsible for IHA activity is present in the preparation (Gilmour, 1980).

The envelope of Gram-negative bacteria is a complex structure and has been the subject of numerous reviews (Costerton, 1974, Rogers et al, 1981). The vast majority of reports describe the structure found in the Enterobacteriaceae. The morphology of the envelope can be determined by physical and chemical methods and can easily be visualised by electron microscopy (Costerton, 1979). Physical methods necessitate breakage of the cell and this can be done by mechanical means, by sonic disintegration or by treatment in a pressure cell. This is usually followed by differential centrifugation. An alternative is enzymatic lysis but this may denature or completely destroy the structures under investigation. Electron microscopy offers a variety of procedures but these are fairly restricted in their power to analyse the morphology allowing only visual examination. Scanning electron microscopy (SEM) can be used to look at surfaces of intact bacteria while transmission electron microscopy (TEM) allows a greater, much closer and detailed observation of thin sections of the cell envelope to be made. It is from TEM studies that valuable information on the structure on the cell wall has been obtained. Freeze etching has been used to look at the fine detail of the cell wall, especially at the layered composition of this structure with its two membranes sandwiching a denser staining layer.

Electron microscopy shows the Gram-negative cell wall to be

composed of an inner trilamellar cytoplasmic membrane enclosed by a dense staining peptidoglycan band, which is in turn enclosed by an outer trilamellar membrane. There may also be a further layer of loosely attached material. In addition, certain appendages such as flagella, fimbriae or pili may be present. Between the inner and outer membrane a gap known as the periplasmic space is found which has an important function in the Gram-negative bacterium, keeping enzymes and other cell products in close proximity to the cell. The Gram-negative envelope is represented diagrammatically in Figure 1.

#### Cytoplasmic membrane

This can be obtained by the method of Miura and Mizushima (1968, 1969) which was later modified by Osborn et al (1972) who used ethylene diamine tetra acetic acid (EDTA) and lysozyme in combination to disrupt the outer membrane and break down the peptidoglycan. This released spheroplasts which were lysed and the outer membrane and cytoplasmic membrane separated by sucrose density centrifugation.

The cytoplasmic membrane is approximately 7.5nm in width and its structure is a bilayer of phospholipid with proteins intimately associated with it. Approximately 40-70% of the membrane is made up of protein with lipid accounting for 10-35%. This membrane is similar to the plasma membrane of eukaryotic cells in that the membrane lipids consist mainly of phosphatides but differs in that they contain no sterols (except for certain mycoplasmas). The protein in this membrane may account for up to 20% of the total protein of a bacterium (Leive and Davis, 1980).

Figure 1

Schematic representation of the Gram-negative cell envelope.

LPS, lipopolysaccharide.

P, protein.

LP, lipoprotein.

PL, phospholipid.

CAPSULE

LPS

OUTER MEMBRANE

PERIPLASM

PEPTIDOGLYCAN

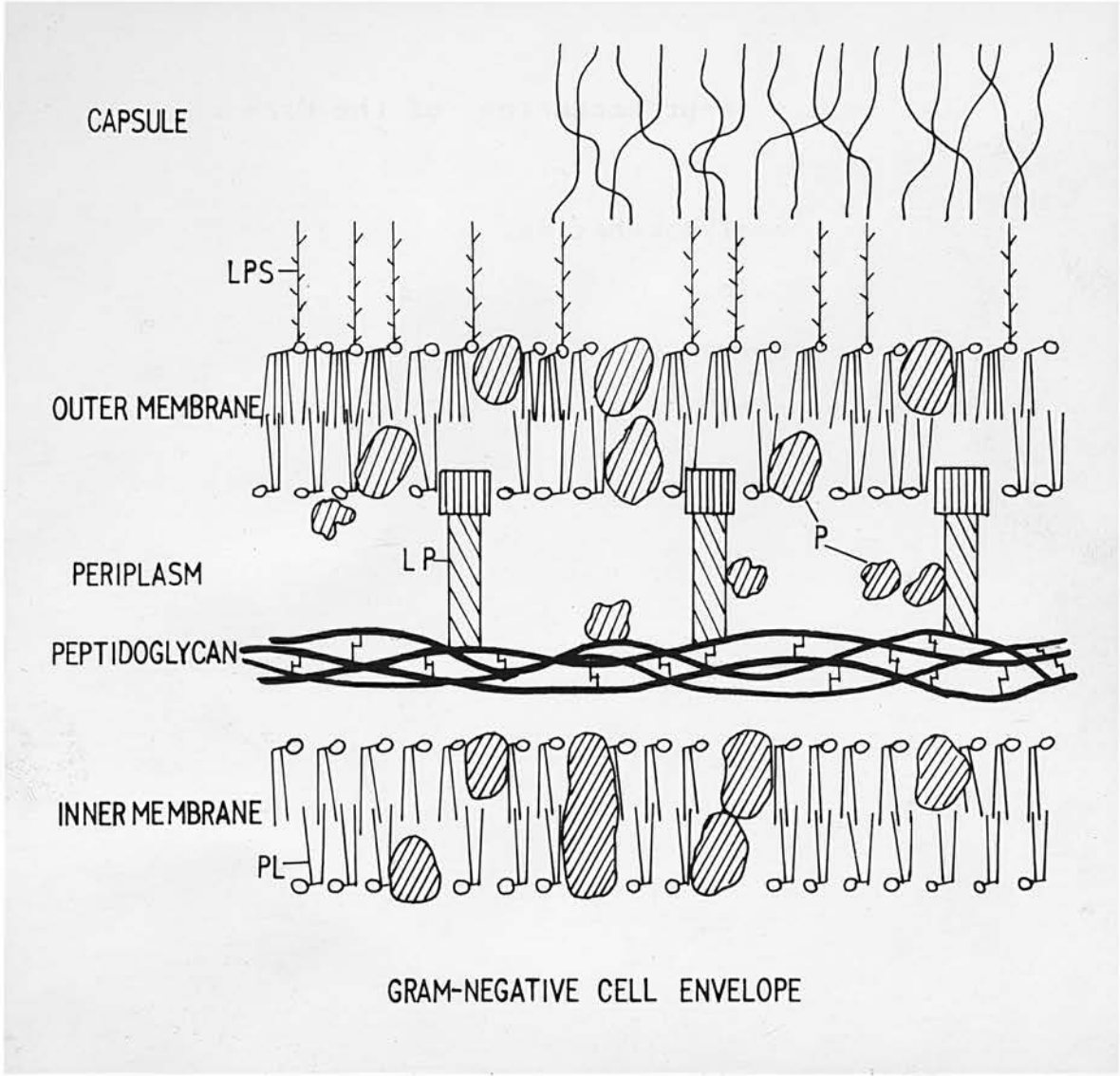
LP

P

INNER MEMBRANE

PL

GRAM-NEGATIVE CELL ENVELOPE





The membrane provides a osmotic barrier for the bacterial cell and the proteins in the membrane are mainly enzymatic in nature although some may provide sructural functions. The enzymatic processes include the specific transport systems, respiratory chain system, lipid biosynthetic processes, wall biosynthesis , DNA replicase and exoenzymes not yet released to the exterior (periplasmic space).

#### Peptidoglycan layer (rigid layer)

The dense layer seen in EM, the peptidoglycan or rigid layer, was first isolated by Weidel et al (1960) using NaOH and sodium dodecyl sulphate (SDS) to disrupt the cells and phenol to remove lipid and protein components. The insoluble material remaining after this was treated again with SDS to remove any residual soluble material. The resultant material, about 10% of the cell mass, contained all the constituents of the peptidoglycan complex. If proteases were used the covalently bound proteins were removed leaving only the backbone components. The unique nature of the marine pseudomonad B16 enabled the outer membrane to be separated from the peptidoglycan by NaCl+SDS+trypsin (Forsberg et al, 1970).

Peptidoglycan is found in all prokaryotic organisms and can be regarded as an indicator for this group. There is a difference in the amount of peptidoglycan contained in the cell walls of Gram-positive and Gram-negative bacteria. In Gram-positive bacteria the peptidoglycan layer is 15-50nm thick while in Gram-negative bacteria the peptidoglycan is almost a monolayer 3-8nm thick.

The composition of this layer is more uniform in

Gram-negative bacteria than it is in Gram-positive bacteria with all Gram-negative bacteria investigated so far having peptidoglycan composed of a glycan chain with peptide substituents. The glycan chain, composed of alternating units of N-acetyl glucosamine and N-acetyl muramic acid, has a peptide chain attached to it through the 3 carbon of the N-acetyl muramic acid. This peptide chain contains D and L isomers of amino acid residues and it is the cross-linking of these chains which gives the structure its mesh like appearance and rigidity.

This rigidity is necessary for cell shape and also as an anchoring point for the layers external to it (Ghuysen, 1968).

#### Outer membrane.

The layer immediately external to the peptidoglycan is the outer membrane. Studies on this layer have concentrated mainly on those found within the Enterobacteriaceae especially those of E.coli and Salmonella typhimurium.

The major difficulty encountered when isolating outer membrane is the separation of this membrane from the other layers in the envelope. The method of Osborn et al, 1972 has already been described in the section dealing with cytoplasmic membrane.

The finding that Triton X100 solubilizes cytoplasmic membrane (De Pamphilis and Adler, 1971) was used by Schnaitman to isolate outer membrane from E.coli (1971). A much more rapid method was devised by Wolf-Watz et al (1973) working with E.coli. After treatment with lysozyme and EDTA,  $MgCl_2$  ribonuclease and deoxyribonuclease are added. Following centrifugation at

48,000g the pH is lowered to pH5 and agglutination occurs giving rise to a precipitate which can then be deposited and washed with distilled water. Poxton and Brown (1979) described a convenient method for the extraction of outer membrane components from Bacteriodes fragilis involving the use of 10mM EDTA and sonication followed by centrifugation of the cells to leave outer membrane components in the supernate.

The outer membrane is composed of three main constituents; phospholipid, protein and lipopolysaccharide. These are arranged as shown in Figure 1. Basically the structure is a lipid bilayer with proteins embedded in it and these proteins can be either located in one half of the bilayer or traverse the whole membrane. The distribution of these components within the outer membrane is interesting and Nikaido and Nakae (1979) calculated that the outer surface of the membrane is 59% protein and 41% LPS while the inner surface has a composition of 53% phospholipid and 47% protein.

**Phospholipids:** The phospholipids in the outer membrane of Enterobacteriaceae are apparently identical to those in the cytoplasmic membrane and thus contain mostly phosphatidyl ethanolamine, some phosphatidyl glycerol and very small amounts of cardiolipin (Osborn et al, 1972). This similarity is more easily understood in the light of findings by Jones and Osborn (1977) that phospholipids move rapidly between outer and inner membranes.

**Proteins:** The proteins of the outer membrane have been the subject of intense study and a number of reviews are in the literature (Nikaido and Nakae 1979; DiRienzo et al, 1978; Rogers

et al, 1981). The investigation of protein components of the outer membrane was advanced rapidly by the application of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to the problem of visualising and resolving the proteins. Work by Ames (1974) and Bragg and Hou (1972) showed that between 30 and 60 protein bands could be identified in the outer membranes of E.coli and S.typhimurium of which 4 or 5 appeared to be major components. Although the greater part of the information on outer membranes has been obtained from these two organisms the result of investigation into other species such as Ps.aeruginosa, and N.gonorrhoeae have shown similarities in that 4-5 major proteins are always present. (Booth and Curtis, 1977, Heckels, 1977).

The major proteins in the outer membrane of E.coli have been isolated and studied and will be described briefly.

The matrix proteins have a molecular weight of approximately 36.5K and are thought to be the constituents of "porins"- pores in the membrane allowing passage of substances from the outside into the cell (Nakae, 1976). They are also implicated as phage receptors.

The Tol G protein, 27-48K, is trypsin sensitive and heat modifiable in that its appearance in SDS-PAGE changes after heating at 50°C.

The lipoprotein, first described by Braun and Rehn (1969) has a lower molecular weight than the two previously described proteins at 7K but it is probably the most abundant protein in the outer membrane. This protein is thought to anchor the outer membrane as it is covalently bound to the peptidoglycan.

Minor proteins, numbering around 10-20, vary in quantity according to growth conditions and would appear to be involved with the uptake of nutrients.

Lipopolysaccharide (LPS): The third component of the outer membrane is LPS. As its descriptive name indicates it is a complex consisting primarily of polysaccharide with a significant amount of lipid attached to, or associated with it.

The LPS is a specific component of the outer membrane and work by Muhlradt and Golecki (1975) has shown that this molecule is located exclusively on the outer surface of the outer membrane of S.typhimurium. In the specific case of S.typhimurium, Smit et al (1975) calculated that there are approximately  $2.5 \times 10^6$  molecules of LPS per cell and this occupies about 45% of the outer surface.

The LPS of the Gram-negative bacterium is the endotoxin and because of interest in the biological effects of this molecule its chemistry, structure and function have been extensively researched (Landy and Braun, 1964; Kass and Wolf, 1973; Rowley, 1971; Westphal, 1975; Kabir, 1978; Wilkinson, 1977; Orskov et al, 1977; Reaveley and Burge, 1972 and Wright and Kanegasaki, 1971).

The accepted general structure for LPS is that found in the Enterobacteriaceae and depicted by Luderitz et al (1971).

O specific side chain - core region - Lipid A.

The molecule is composed of three covalently linked segments each with its distinctive composition, biosynthesis and biological function. The side chain is the serologically

dominant part of the molecule responsible for its O-antigenic specificity and consisting of repeating oligosaccharides. R-form lipopolysaccharides are deficient in the side chain and are present in rough strains. The core region contains sugars not found elsewhere in the bacterium including a 3-keto deoxyoctonic acid and an aldoheptose. The core is linked to lipid A which also contains distinctive components such as glucosamine and 3-hydroxy alkanolic acids. In contrast to the variety that exists in "O" side chain structure within a genus the core and lipid A are quite conserved (Luderitz et al, 1966).

The entire lipopolysaccharide molecule is thought to have a molecular weight somewhere between  $1 \times 10^6$  and  $20 \times 10^6$  (Kabir et al, 1978) and the reason for the wide ranging limits are two fold. The first is that lipopolysaccharides are amphipathic molecules containing both hydrophilic (polysaccharide) regions and hydrophobic (Lipid A) regions and when suspended in aqueous solution they tend to form aggregates (micelles), probably due to hydrophobic forces. The second is that the "O" side chain oligosaccharide has been shown to be heterogeneous in length in salmonellae (Nowotny, 1966) and E.coli (Morrison and Leive, 1975). The amphipathic nature of the molecule is evident in its orientation in the membrane with the lipid A intercalating with the lipid and the "O" antigen chain projecting out of the cell. As with outer membrane proteins studies on the Enterobacteriaceae lipopolysaccharides have provided the vast majority of the published reports.

A number of methods have been used for the extraction of lipopolysaccharides most of which involve the treatment of the



bacterial cells with organic reagents such as trichloroacetic (Boivin and Mesrobianu, 1933) aqueous pyridine (Goebel et al, 1945) and aqueous ether (Fukushi et al, 1964). However in addition to the lipopolysaccharide all of these methods also remove other cell wall components such as phospholipids, lipoprotein and protein. The most common method used for preparing lipopolysaccharides is the phenol-water method of Palmer and Gerlough (1940) which was later modified by Westphal et al (1952). This procedure involving extraction at 68°C with phenol-water (45:55v/v) gives relatively pure lipopolysaccharides which can then be centrifuged out of solution. Galanos et al (1969) modified this procedure still further to allow the extraction of rough lipopolysaccharide which, due to their loss of "O" side chains, are less hydrophilic. This extraction is carried out at room temperature with a mixture of phenol/chloroform/ether.

A method for isolating lipopolysaccharide which did not use organic reagent was described by Galanos et al (1975) where electrodialysis produced a soluble form of lipid A.

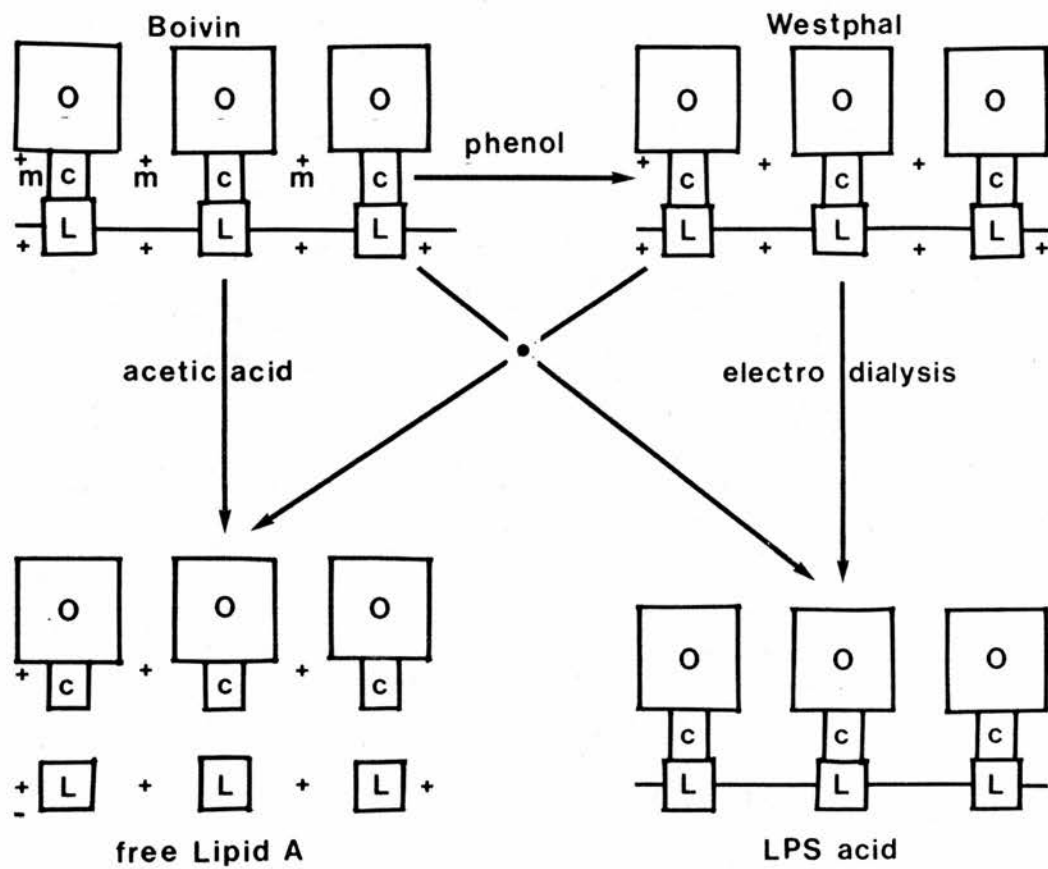
Figure 2 shows the schematic representations of various types of lipopolysaccharide preparations (figure from Bradley, 1979).

Most structural studies on lipopolysaccharides have involved degradation of LPS by partial acid hydrolysis with 1% acetic acid (Schmidt et al, 1969) followed by separation on Sephadex G50 which in the case of 'smooth' lipopolysaccharide results in 3 distinct fractions: "O" antigen, core and KDO. The

**Figure 2**

Schematic representations of various types of lipopolysaccharide preparations. O, somatic antigens; C, heterooligosaccharide core; L, Lipid A; m, mitogenic peptide; +, metal cations and polyamines. (taken from Bradley, 1979).





combination of gas chromatography and mass spectrometry allowed a more detailed examination of composition and structure of the "O" antigen side chain and it is now known that common hexoses (L-rhamnose, L-fucose ) and hexosamines, (D-glucosamine, D-galactosamine), are widely distributed in bacterial lipopolysaccharides while pentoses (arabinose, ribose, xylose and uronic acids (glucuronic, galacturonic) are less frequent. A large number of unusual sugars are detailed by Wilkinson (1977) and includes such sugars as 3,6-dideoxy D-galactose (abequose), 3,6-dideoxy mannose (tyvelose) and 3,6-dideoxy L-galactose (colitose) which are present in the LPS of salmonellae and in some Yersinia species.

In the "O" antigen side chains there is potentially great variety in the arrangement of the substituent sugars which can be linear or branched in the structure. This gives correspondingly great variety in the antigenic nature of the molecule which is probably an important function of the "O" antigen side chain. Within a single genus this can give a remarkable number of different serotypes (Orskov et al, 1977).

The core regions are attached to the repeating oligosaccharide of the "O" antigen side chain and contain hexoses (glucose, galactose) , heptose (L-glycero, D-manno heptose) keto-deoxyoctonic acid and amino sugars such as N-acetyl glucosamine. Phosphates and phosphatidyl ethanolamine are also found in the core.

In Salmonella species the core grouping is highly conserved with only one core composition throughout the genus while in E.coli and Shigella species a number of different arrangements

are found (Wilkinson, 1977). The genus Bacteroides differs from this general structure for Gram-negatives in that they do not contain heptose or keto-deoxyoctonic acid which are generally regarded as specific markers for lipopolysaccharide (Wilkinson 1977). Ketodeoxyoctonic acid and heptose have been detected by Schmerr and Rebers (1979) in P.multocida indicating that the lipopolysaccharide is probably similar to those in the Enterobacteriaceae.

Lipid A is connected to the polysaccharide regions by an acid labile link with keto-deoxyoctonic acid, and can be isolated as the insoluble product after mild acid hydrolysis with 1% acetic acid. In salmonellae the back bone of Lipid A is a B 1,6-linked disaccharide of glucosamine. The bulk of the lipid A structure is composed of fatty acids which are attached to the glucosamine via ester and amide linkages. B-hydroxy acids are found in Lipid A but are not present in other cellular lipids making them useful markers for this molecule. However, not every Gram-negative organism contains these acids and Nikaido and Nakae (1979) indicate that Brucella species are reported not to have any. Phosphorylation in Lipid A is thought to be responsible for the aggregation of monomeric units into larger molecules, 4-5 units in size (Luderitz et al, 1973).

Lipopolysaccharide is a complex molecule having numerous biological effects most of which can be assigned to the Lipid A portion. These include pyrogenicity, lethal toxicity, bone marrow necrosis and leukopenia. The polysaccharide regions, especially the "O" antigen side chains help to protect the bacterium both physically and immunologically. The long chains

inhibit phagocytosis and act like a picket fence while the vast number of possible configurations allows the bacterium to avoid the host immune system.

### Capsule

Many Gram-negative bacteria produce an external layer composed of extracellular polysaccharide. This may take the form of a discrete capsule or may appear as a loosely attached slime layer which is easily lost into the culture media. The removal of capsule, however, usually requires vigorous shaking or treatment with alkali.

Capsules can be visualised by light microscopy using the thin indian ink method Duguid (1951) or the congo red, lactophenol blue method of Maneval (1941). As most capsules are negatively charged they may be stained with ruthenium red (Howard and Gourlay, 1974) or labelled with cationic ferritin (Weiss et al, 1979) and examined by electron microscopy.

The composition of exopolysaccharide is usually simpler than that of lipopolysaccharide with neutral hexoses such as D-glucose, D-galactose and D-mannose frequently present. Methyl pentoses (6 deoxy hexoses) such as L-fucose and L-rhamnose and polyols, (ribitol and glycerol) are also frequently identified. Uronic acids contribute towards the negative charge often found in exopolysaccharides.

Pentoses, heptoses and octonic acids are rarely found in capsules. However, non sugar components are found and phosphate, acyl groups, pyruvate, formate and succinate have been described (Sutherland, 1977).

The exopolysaccharides can be divided into two main groups:

## 1, homopolysaccharides and 2, heteropolysaccharides

1. Homopolysaccharides are composed of one sugar and are usually very long chain molecules. Cellulose, levans (fructose polymer) and glucans (glucose polymer) are examples of this type. Linkage differences within glucose residues gives rise to different glucans and 1-→6, 1-→3 and B1-→2 linkages are found. Less frequently encountered is the bacterial cellulose (B 1-→4) which is found in Acetobacter xylinum. Levans have been isolated from Xanthomonas, Aerobacter and Acetobacter species.

Another type of homopolysaccharide is the sialic acid polymer (N-acetylneuraminic acid) found in some E. coli strains and Neisseria meningitidis (references cited in Sutherland, 1977).

2. Heteropolysaccharides are more complex structures composed of 2-4 monosaccharides and frequently containing uronic acid. The use of paper and gas chromatography was a great advance in the analyses of these structures. Some *Klebsiella* capsules are well defined and components such as pyruvate, glucuronic acid, rhamnose, fucose, mannose, glucose, galactose and O-acetyl groups have been found in them. It has also been shown that different strains in one serotype may have different capsule structures (references cited in Sutherland, 1977).

Hyaluronic acid, a heteropolymer of N-acetyl glucosamine and glucuronic acid is a component of both prokaryotic and eukaryotic cells.

The capsule is very hydrated being only 1-2% polysaccharide

and probably functions as a moisture and ion trapping layer around cells as well as an inhibitor of phagocytosis.

### Bacterial cell surface antigens

The envelope is the part of the Gram-negative bacterium which interacts directly with the host and because of this the components of the envelope contribute an important antigenic stimulus to the host. The antigenicity (the reactivity with antibodies) and the immunogenicity (the capacity to induce the formation of antibodies in mammals) of the surface components are subjects of great interest to bacteriologists looking at the structure and function of the bacterial cell surface as well as its role in pathogenesis.

A clear indication of the complex antigen mosaic presented by the Gram-negative bacterium is given by the large number of precipitin lines present when envelopes are examined by crossed immunoelectrophoresis (CIE). This involves two dimensional electrophoresis which separates antigens by their charge in the first dimension then visualises them by precipitation against specific antiserum in the second dimension (Weeke, 1973b). When the envelope of E.coli was examined by this method at least 25 immunoprecipitates were visible in the outer membrane portion (Smyth et al, 1978). The LPS and Braun lipoprotein were identified as the major antigens. More recently Bhasin and Lapointe-Shaw (1980) have shown that when P.multocida envelopes are examined in CIE at least 19 antigens can be counted. No attempt was made to identify individual immunoprecipitates.

In an examination of the outer membrane of N.meningitidis group B 7 different precipitin lines were observed, among which the capsular and LPS immunoprecipitates could be distinguished (Hoff and Frasch, 1979).

Polysaccharides are antigenic components of the envelope and the capsular ("K") or LPS ("O" and "R") antigens are the ones mainly responsible for the immunological individuality of the Gram-negative bacterium. These two types of antigen can occur in the same cell and the immunological response can be governed by the interaction of these two. Good reviews on polysaccharide antigenicity have been published (Jann and Westphal, 1975; Jann and Jann, 1977; and Orskov et al, 1977).

As many of these components are very antigenic they tend to be the dominant surface antigen and the large classification schemes for *Salmonella* species and *E.coli* are based on the agglutination reactions of the antigens with specifically absorbed antisera (Kauffman, 1954; Orskov et al, 1977).

The IHA test for serotyping *P.haemolytica* isolates is thought to depend on a soluble capsular antigen (Biberstein et al, 1960), and more recently Frank and Wessman (1978) have shown that the species can also be serotyped by a plate agglutination test which suggests that the serotyping antigen is a polysaccharide and present on the surface of the bacterium.

The enzyme - linked immunosorbent assay (ELISA) developed by Engvall and Perlmann (1972) although primarily for measuring antibodies has also been used to examine the surface antigens of Gram-negative bacteria. Poxton (1979) has shown that *Bacteroides* species are related through the antigens present in EDTA extracts of the cell envelopes when these are tested in an ELISA, and the method has been applied to the detection and quantitation of the capsular antigen of *H.influenzae* type b (Crosson et al, 1978).



Other serological methods have been employed to analyse the antigens of bacterial cell surfaces including agar gel diffusion for P.multocida antigens (Penn and Nagy, 1976), simple immunoelectrophoresis LPS and proteins of Yersinia enterocolitica (Ogata et al, 1972) and radio-immune assay on H.influenzae type b outer membrane proteins (Hansen et al 1981).

The immunogenicity of the cell surface antigens has been studied most carefully as it is a property which may be important in conferring protection against infection. Although bacterial polysaccharides are often very antigenic their immunogenicity is dependent on such things as molecular weight, the dose of polysaccharides given and their interaction with the immunological mechanism of the host (Jann and Jann, 1977).

The type of problem which can be encountered is well documented for the work carried out on Neisseria meningitidis. Serogroup specificity of N.meningitidis is dependent on the capsular polysaccharide antigen. The capsular antigens of groups A,B and C have been isolated and purified and tested as vaccines. The antigens for groups A and C have been used successfully in vaccines but in group B the capsular polysaccharide is not the antigen important in protection (Goldschneider et al, 1969). It has since been shown that in the B group the outer membrane protein of 41,000 molecular weight is involved in protection (Steele et al, 1977).

The studies on P.haemolytica have yielded conflicting results as to the important antigens involved in protection. As stated earlier in this review, Biberstein and Thompson (1965) concluded that both surface antigens and capsular antigens were

involved but that the capsular antigen was more important. Knight et al (1969) subsequently repeated the work but found that immunity did not correlate with capsular type. Cameron (1966) reported that both protein and polysaccharide antigens were implicated in the passive protection of mice against challenge. More recently both Gilmour et al (1979) and Evans (1979) have shown that SSE extracts, presumably containing surface material have been protective in both sheep and mice.

Protein antigens have been shown to protect against E.coli B infection in mice (Taplits, 1979) and Lam et al (1980) working on H.influenzae type b demonstrated that even after antisera had been absorbed with capsular antigen the antisera still passively protected. This indicated that other antigens were involved. The proteins which have been described as porins in the outer membrane have also been used in vaccines. The porins of Salmonella typhimurium were protective against challenge with the organism (Kuusi, 1979) and LPS was shown not to be necessary for protection.

As the potency of an antigen as an immunogen can be lost on isolation and purification it can prove difficult to assess the immunogenicity in pure form. An elegant answer to this problem was provided by Diena et al (1979). An E.coli hybrid expressing Salmonella typhi somatic antigens 9 and 12 and a derivative expressing these two plus the capsular antigen Vi were compared with the parent S.typhi strain against challenge with S.typhimurium. The hybrid expressing only somatic antigens was the more effective of the two hybrids but was not as good as the parent strain. It is interesting that the inclusion of the Vi

antigen did not improve the protection.

Penn and Nagy (1976) have shown that P.multocida capsular antigens stimulated mouse-protecting antibodies in cattle. The difficulties related to immunogenicity were illustrated here as the polysaccharide did not stimulate antibodies in rabbits but was effective in cattle.

The foregoing section on immunogenicity serves to emphasize the complex interaction of bacterial surface antigens and the host's immune system. It suggests that a simple relationship between one antigen and protection is an infrequent event and that immunity to bacterial infection is much more likely to be multifactorial.

## Materials and Methods

### Bacteria

The serotype strains of P.haemolytica used in this study were taken from the stock prototype strain collection kept at the Moredun Research Institute (MRI). These strains were stored at  $-70^{\circ}\text{C}$  in No.2 nutrient broth (Oxoid Ltd., Basingstoke, Hampshire). Strains of serotypes A1, A2, A6, A7 and A9 were all recovered from cases of sheep pneumonia.

Strains of P.haemolytica referred to the MRI for serotyping but which failed to react in the IHA test were designated untypable strains. These were subcultured into 50 ml of nutrient broth and grown for 18 h at  $37^{\circ}\text{C}$  without agitation after which 1 ml aliquots were dispensed into 2 ml vials and stored at  $-70^{\circ}\text{C}$  till required.

Culture conditions: Unless otherwise stated the bacteria were removed from  $-70^{\circ}\text{C}$  storage, thawed and plated out on 7% sheep blood agar plates which were then incubated at  $37^{\circ}\text{C}$  for 18h. Single colonies were picked from these plates and inoculated into 50 ml nutrient broths which were incubated at  $37^{\circ}\text{C}$  for 18h. If required this was used as a seed broth for larger volumes. Large quantities of bacterial cells for extraction were produced by inoculating 1.5L volumes of nutrient broth with 15ml of seed broth. These were incubated at  $37^{\circ}\text{C}$  for 6 or 18 h on an orbital shaker (LH Engineering, Stoke Poges, Bucks.). Bacteria were also grown on blood agar base (Oxoid) supplemented with 1% galactose (Wessman, 1966).

### Preparation of antigens

Sodium salicylate extract(SSE): Cells of P.haemolytica were extracted with 1M sodium salicylate. Cells from 3l of nutrient broth culture incubated at 37°C for 6h on an orbital shaker, were harvested by centrifugation at 4200g for 45 min on an MSE Coolspin centrifuge(MSE, Crawley, Sussex). The cells were resuspended in 300ml of 1M sodium salicylate, shaken vigorously for 3h at 37°C and then centrifuged at 20000g for 30 minutes to remove the cells. The supernate was further clarified by centrifugation at 40000g after which it was concentrated by ultrafiltration in an Amicon 200ml cell containing an XM 100A membrane(Amicon, Lexington, USA) under 12 pounds per square inch (psi) compressed air pressure. The concentration was stopped when the volume in the cell reached 30 ml. The concentrate was removed and dialysed against two changes of phosphate buffered saline(PBS, 0.02M sodium phosphate, 0.03M sodium chloride, pH 7.6) and two changes of distilled water. The dialysed extract was dispensed in 5ml volumes and lyophilised till required.

Ethylene diamine tetra acetic acid (EDTA) extract: P.haemolytica

cells grown in nutrient broth for 18h at 37° were harvested by centrifugation at 4200g for 45 min, washed twice in PBS and then lyophilised. Five grammes dry weight of cells were then resuspended in 50ml of 10mM EDTA in buffer and shaken vigorously at 45°C for 30 minutes. The bacterial cells were then sheared by passing the suspension twice through a 23 gauge needle. The cells were removed by centrifugation at 12000g. The supernate was

dialysed against two changes of distilled water for 24h then lyophilised.

Saline extract: P haemolytica cells grown on blood agar plates for 18h at 37°C were harvested by flooding the plate with 5ml of PBS and gently scraping the surface of the agar with a bent sealed pasteur pipette. This suspension was washed once in PBS, resuspended in 5ml PBS and 0.5 ml volumes used to seed 10 six inch diameter glass petri dishes containing 60ml of 1% galactose agar. These plates were incubated for 18h at 37°C. The cells were harvested in 2.5% saline using the method described above. The suspension was then homogenised on an MSE mixer for 5 minutes at full speed after which the cells were removed by centrifugation at 3000g on a bench centrifuge. The supernate was filtered through a 0.45um Millipore filter (Millipore, London). The resultant translucent supernate was precipitated with four volumes of cold acetone (-20° C) and left overnight in the ice box of the refrigerator. The precipitate was washed once in acetone then lyophilised till required.

Phenol water extract (PWE): The method described by Westphal et al (1952) was used to extract dried cells of P. haemolytica. Cells grown in nutrient broth for 18h at 37° C were harvested by centrifugation at 4200g for 45 min, washed twice in distilled water, then lyophilised. Dried cells were resuspended in 50ml of distilled water and mixed with an equal volume of 90% (w/w) phenol at 68°C. The mixture was shaken for 10 min at 68°C, cooled quickly in an ice bath before centrifugation at 8000g for 20 min to deposit the cells. The phases of the mixture were separated after cooling and centrifugation, the upper aqueous layer was

removed ,without disturbing the interface,and dialysed against running water for 24h before lyophilisation.SSE's of P.haemolytica serotypes were further extracted with hot phenol water by mixing equal volumes of phenol and SSE in aqueous suspension ( $2.5\text{mg ml}^{-1}$ )at  $68^{\circ}\text{C}$  and proceeding in the same manner as that described for whole cells.

Heated culture extract(HE): P haemolytica cells,grown on 7% sheep blood agar for 18h at  $37^{\circ}\text{C}$ ,were harvested and resuspended in 1ml of PBS.The suspension was heated at  $56^{\circ}\text{C}$  for 45min,centrifuged at  $11500g$  for 10 min and the supernate stored at  $-20^{\circ}\text{C}$ .

Heat killed organisms(HKO): P haemolytica cells from 1 litre nutrient broth culture incubated at  $37^{\circ}\text{C}$  fo 18h were harvested by centrifugation at  $4200g$ ,washed once in PBS,and finally resuspended in 10 ml PBS.A 0.1ml aliquot was removed for viable counting and the remaining 9.9ml heated at  $56^{\circ}\text{C}$  for 1h.Another 0.1ml aliquot was taken to check for sterility before the suspension was lyophilised.

Sequential saline and phenol-water extraction of cells grown on galactose agar: P.haemolytica A1 cells grown on 3 petri dishes of 1% galactose agar for 18h were treated as described by Penn and Nagy (1976) .Cells were harvested in 2.5% saline,agitated on a wrist action shaker (Stuart Scientific, Croydon ) for 30min and the cells removed by centrifugation at  $3000g$ .The supernate was dialysed against running tap water for 24h before lyophilisation.This was regarded as the saline(pg)extract.The cells recovered at centrifugation were extracted with hot phenol-water as described for PWE.



Treatment of Al PWE.

- 1) Sodium periodate ( $\text{NaIO}_4$ ): PWE (2mg) was resuspended in 2ml of 50mM  $\text{NaIO}_4$  in acetate buffer pH 5. This was left in the dark at RT for 18h then dialysed against running tap water for 24h.
- 2) Proteinase K: Proteinase K (Sigma Chemical Co., Poole, Dorset) was added to Al PWE to give a final concentration of  $1\text{mg ml}^{-1}$  for PWE and  $10\text{ug ml}^{-1}$  proteinase K in PBS (pH 7.4). This was incubated at RT for 24h, stopped by heating at  $70^\circ\text{C}$  for 30min and then dialysed against running tap water for 24h.
- 3) Heat: Al PWE at  $1\text{mg ml}^{-1}$  was autoclaved at  $120^\circ\text{C}$  at 15 psi for 20 min. The sample was dialysed against running tap water for 24h.
- 4) Partial hydrolysis: PWE (10ug) was hydrolysed with 1% acetic acid at  $100^\circ\text{C}$  for 90 mins in a sealed test tube and the resulting precipitate was removed by centrifugation at 50000g for 10 mins. The supernate was rotary evaporated to dryness and resuspended in 1ml distilled water. The precipitate (presumed to be Lipid A) was washed once then freeze dried.



## Sera

Rabbits: New Zealand white rabbits were hyperimmunised with repeated doses of formalin killed cells of P.haemolytica. Rabbits were immunised according to the schedule in Table 2.

One week after the final dose the rabbits were bled from the ear vein and the sera tested by the IHA test. If the titre was satisfactory, usually  $>1$  in 128 then the rabbit was exsanguinated by cardiac puncture. If the titre was unsatisfactory then the rabbit was given a further two 3ml doses of live organisms i.v.. This was always the case with serotype A2. If this failed to raise the titre of the antiserum then the attempt to immunise was stopped.

Rabbit IgG was prepared by fractionation on protein A-Sepharose CL-4B (Pharmacia, (Great Britain) Ltd., Hounslow, Middlesex) by the method of Goding (1976) followed by dialysis against PBS. The IgG was dispensed into 2 ml aliquots and stored at  $-20^{\circ}\text{C}$ .

Sheep. Antisera to serotypes A1, A6 and A7 were obtained from specific pathogen free (SPF) lambs vaccinated with individual SSEs ( $1.1\text{mg ml}^{-1}$  for A1 and A6 and  $5.5\text{mg ml}^{-1}$  for A7) in alhydrogel/oil as described by Wells et al (1979). Antisera to serotypes A2 and A9 were obtained from SPF lambs which had been vaccinated with A2 or A9 SSE then challenged with the homologous serotype using the aerosol method described by Gilmour et al (1979)

## Serology

Indirect haemagglutination (IHA) test: For antibodies against P.haemolytica, serum samples were tested by the method described by Shreeve et al (1972). Antigen was prepared from a nutrient broth culture of P.haemolytica incubated at

Table 2 Hyperimmunisation schedule for the production of hyperimmune antisera against P haemolytica serotypes in rabbits.

Day	Dose
1	0.5ml s.c (1x10 <sup>6</sup> cfu ml <sup>-1</sup> )
4	1.0ml "
7	2.0ml "
10	2.0ml i.v
13	3.0ml "
16	3.0ml "
19	3.0ml "
22	3.0ml "
25	3.0ml "

s.c=subcutaneously  
i.v=intravenously

37°C for 18h. The culture was heated at 56°C for 45 minutes after which bovine RBC, fixed in 1% glutaraldehyde (Shirai et al, 1975), were added to a final concentration of 0.5% and incubated at 37°C for 1h. The RBCs were washed three times in formalised phosphate buffered saline (FBS) (0.3% formalin), and resuspended at 0.5% in the same solution. Doubling dilutions of test sera were made in FBS in U bottomed microtitration plates (Cooke Engineering Company, Alexandria, Virginia, USA). Equal volumes (0.025ml) of sensitised RBCs were added to each well and left for 2h at room temperature. The titre of the sera was the highest dilution at which the RBCs were agglutinated.

For antigen the samples to be tested were diluted in FBS in ten fold dilutions from 1 in 10 to 1 in 100,000 in 2ml volumes. Glutaraldehyde fixed RBCs were sensitised by adding 0.2 ml of a 5% washed suspension to the sample. The tubes were incubated at 37°C for 30 min, the cells washed three times in FBS and then resuspended to their original volume. An homologous rabbit antiserum at a previously determined optimal dilution, was dispensed in 0.025ml volumes into the wells of a microtitre plate. After the addition of 0.025ml of sensitised RBC suspensions to the appropriate wells the plate was left at RT for 2h before examination for haemagglutination. Positive and negative controls were included and the highest dilution of sample which produced haemagglutination was the IHA titre of the sample.

Enzyme linked immunosorbent assay (ELISA): The technique was a modification (Burrells et al, 1978) of that described by Engvall and Perlmann (1972). P. haemolytica antigen in 0.05M

carbonate/bicarbonate (CB) buffer pH 9.6, was used to coat the wells of a polystyrene microtitre plate (type 129A, Dynatech Laboratories Ltd, Billingham, Sussex). Individual wells were filled with 200  $\mu$ l of antigen solution and the plates were held at 4°C overnight. Plates were then washed three times with PBS containing 0.05% Tween 20 (PBS/Tween), shaking out excess fluid between each wash. Standard and test sera were not pre-treated prior to testing and dilutions of these in PBS/Tween containing 0.02% sodium azide were added in 200  $\mu$ l volumes to the wells of antigen coated plates which were incubated at RT for 3h. Serum dilutions were removed from the wells, the plates washed three times with PBS/Tween and 200  $\mu$ l of pig anti-sheep IgG conjugated with alkaline phosphatase diluted in PBS/Tween added to the wells. After incubation for 3h at RT the plates were emptied and washed. Enzyme substrate (p-nitro phenyl phosphate, Sigma Chemical Co., Poole, Dorset) at a concentration of 1 mg ml<sup>-1</sup> in 10% diethanolamine buffer, pH 9.8, containing 0.5M magnesium chloride was added to the plates in 200  $\mu$ l volumes. After 1h at RT the reaction was stopped by the addition of 3M sodium hydroxide. Results were recorded as optical densities at 405 nm (A<sub>405</sub>) and were determined using a "Titertek multiskan" multichannel spectrophotometer (Flow Laboratories, Irvine, Ayrshire). With this test it has been found that A<sub>405</sub> readings below 0.1 are generally unreliable (personal observation) and this A<sub>405</sub> has been arbitrarily adopted as the negative baseline for the ELISAs reported in this thesis.

Double agar gel diffusion: The technique was essentially that described by Ouchterlony (1948). Agarose (1%) in 25% barbital/TRIS/glycine buffer pH 8.8 (Weeke, 1973a) was poured to a thickness of 2mm on the required size of glass plate or Gelbond (FMC Corporation, Marine Colloids Division, Bio-Products, Rockland, Me, USA). Six 2mm diameter wells were arranged circumferentially around a central 2mm well at a distance of 10mm. Antigens and antisera were added to the appropriate wells in 8ul volumes and left at 4° C. The plates were examined at 24h intervals up to 72h and the precipitin pattern if present was noted.

Fused rocket immunoelectrophoresis (FRIE): The method used was that described by Svendsen (1973). Strips of Gelbond, 8x4cm, were covered with a 2mm thickness of 1% agarose in 25% BTG buffer containing antisera at the required concentration. Wells, 2mm, in diameter were punched in two parallel but offset rows. Antigen samples were added to the wells and left to diffuse for 1h at RT, before electrophoresing into the antibody containing gel at 20 volts for 18h. The gels were pressed, washed and then stained with Coomassie blue as described by Weeke (1973a).

Counter current immunoelectrophoresis (CCIE): Strips of Gelbond, 8x8cm, were covered with a 2mm thickness of 1% agarose in 25% BTG buffer. Wells, 2mm in diameter, were punched 5mm apart with 10 mm between parallel rows. Antigen and absorbed antisera were loaded in 8ul volumes into alternate rows. Electrophoresis was 12v cm<sup>-1</sup> with the antiserum row placed nearest to the anode. After 1h the gels were examined and precipitin lines

noted. If required the gels were pressed, washed and then stained with Coomassie blue.

Crossed immunoelectrophoresis (CIE): The procedure was essentially that described by Weeke (1973b). a) First dimension. Samples were applied in wells punched in 1% agarose in 25% BTG buffer (four wells on an 80x80x1.5 mm glass plate). Buffer was used undiluted (ionic strength 0.08) in the electrode reservoirs but in gels and in sample buffer it was diluted 1 in 4 with distilled water. Electrophoresis was carried out at 12.5v  $\text{cm}^{-1}$  for 45 min. After the first-dimension electrophoresis the gel was divided into four slabs, each corresponding to one well. b) Second dimension. Each of the four first-dimension slabs was then transferred to the edge of a 50x50 mm square of Gelbond film and the remaining part of the film was filled with 1% agarose containing antiserum at a previously determined optimal dilution for each antiserum. After electrophoresis for 18h at 4v  $\text{cm}^{-1}$  the gel was pressed, washed and stained with Coomassie blue.

The CIE procedure was modified to include an antibody containing intermediate gel. After the antibody containing gel of the second dimension had set, a 1cm slice across the gel, including the join between first and second dimension gels, was removed. This gap was then filled with 1% agarose, with or without antiserum, and the second dimension run carried out as described.

### Other methods

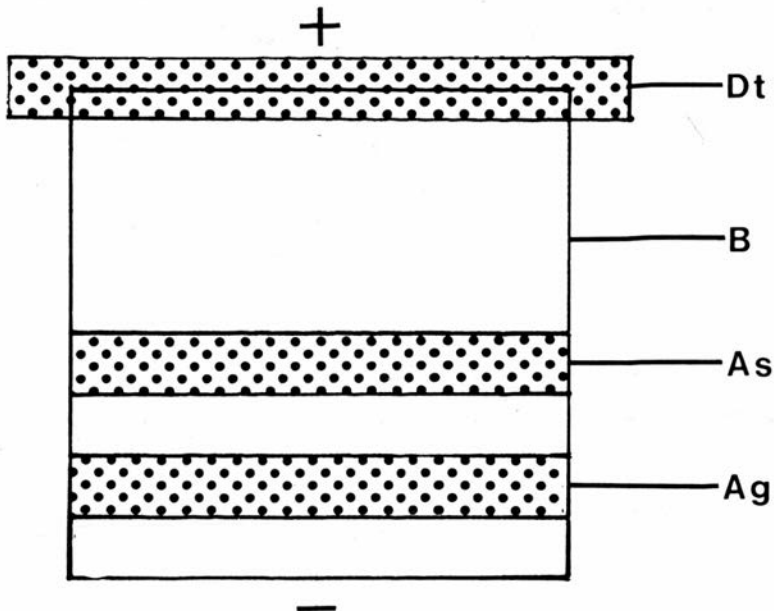
Preparative agarose gel electrophoresis: Agarose (1% in 25% BTG buffer) was poured to a depth of 2 mm on an 80x80x1.5 mm glass plate. A 1cm slice was removed 1cm from one edge and this space filled with 1% agarose containing Al PWE ( $1\text{mg ml}^{-1}$ ). At a distance of 1cm from this insertion another 1cm slice was removed and replaced with 1% agarose containing rabbit antiserum raised against A9 cells. A schematic diagram of the plate arrangement is shown in Figure 3. The gel was electrophoresed as for the second dimension of CIE. A strip of dialysis tubing was placed between the agarose gel and the wick at the anodic end to prevent the unprecipitated antigen running into the wick. After electrophoresis the agarose gel on the anodic side of the antiserum loaded gel was removed and freeze dried. The freeze dried material was crushed then washed three times in distilled water. The supernates from the washings were pooled and this together with the residual freeze dried agarose were stored at  $-20^{\circ}\text{C}$ .

Preparation of immunosorbent column: The IgG fraction of a rabbit antiserum to P.haemolytica A9 was coupled to 10.5ml of swollen CNBr-activated Sepharose 4B (Pharmacia) by the method recommended by Pharmacia in "Affinity Chromatography, Principles and Methods". The immunoabsorbent was packed in a column 10x1cm and equilibrated with 2-3 volumes of 0.1M phosphate buffer, pH 7.0 containing 0.5M NaCl and 0.5% Tween 80 (starting buffer). Al PWE ( $5\text{mg ml}^{-1}$ ) in starting buffer was applied to the column in a 1ml volume and washed with 30ml of starting buffer. Ten 3ml fractions were collected. The flow was reversed and any bound

Figure 3

Schematic representation of the agarose gel for separation of A1 PWE antigens. Ag, A1 PWE antigen; As, rabbit antiserum to A9 whole cells; B, agarose gel; Dt, dialysis tubing.





material was eluted with carbonate/bicarbonate buffer, pH 9.6, in ethylene glycol (50:50 vol/vol). Ten 3ml fractions were again collected after which the column was washed with starting buffer to equilibrate before storage at 4°C.

Paper chromatography: Samples were hydrolysed in 2M HCl at 100°C for 2h and dried several times in vacuo over NaOH pellets and phosphorus pentoxide. The hydrolysates were resuspended in 0.1ml distilled water and together with standards were added in 10ul volumes to the paper. This was allowed to run overnight in a descending chromatographic system with butan-1-ol/pyridine/water (6:4:3; by volume) as solvent on Whatman's No. 1 paper. Reducing sugars were stained by the alkaline silver nitrate method of Trevelyan et al (1950)

Column chromatography: Samples were chromatographed on Sephadex G 50 (Pharmacia, Milton Keynes, Bucks, U.K.) column with distilled water as the mobile phase. The sample (1ml) was washed carefully into the top of the column and run through with a flow rate of 1ml per 5 min. Fractions (1ml) were collected using an LKB Ultrarac 11 fraction collector (LKB, Bromma, Sweden).

Gas chromatography (GC): For the analysis of sugar composition, dry hydrolysates of samples were converted to alditol acetates by a method modified from that of Lindberg et al (1978). The hydrolysate (1-20mg) was dissolved in 1ml of distilled water and 10mg sodium borohydride was added. After 1h at 20°C the borohydride was destroyed by the addition of a few drops of glacial acetic acid. The sample was dried by rotary



evaporation and borate as methyl borate was removed by three distillations with methanol. The dry alditols were acetylated in 1ml pyridine/acetic anhydride(1:1,v/v) at 100°C for 1h. Excess reagents were removed by several co-distillations with toluene in a rotary evaporator and the alditol acetates taken up in chloroform. Samples were examined on columns of 3% OV 225 on Gas-chrom Q in a Pye-Unicam model 104 gas chromatograph, with the temperature controlled between 190° C and 210°C rising by 2°C min<sup>-1</sup>. Alditol acetates of known sugars were run as standards either separate from or co-injected with the test sample.

Other analytical techniques: Phosphorus was estimated by the method of Chen et al (1956); sugars, as glucose equivalents, by the method of Dubois et al (1956); protein by the method of Lowry (1951); heptoses and keto-deoxyoctonic acid (KDO) by the method of Osborn (1963); fatty acids by the method of Duncombe (1963); sialic acid by the method of Cassidy et al (1966) and uronic acids by the method of Dische (1947).

#### Laboratory animal techniques

Mice: Unless otherwise stated, mice used in experiments were of the C57 black strain, bred at the MRI. The mice were of both sexes and were 4-6 weeks old at the the start of experimentation.

Inoculations: 1) Subcutaneous(s.c.) inoculations into mice were performed without anaesthesia. Mice were injected under the loose skin of the groin using a 25 gauge needle. When more than one injection per mouse was required they were given in opposite sides of the groin. 2) Intraperitoneal(i.p.) injections were

also given without anaesthesia using a 25 gauge needle. The injection was into the abdomen, slightly to the left of the umbilicus.

Determination of  $LD_{50}$  for P.haemolytica: The method used was essentially that used by Evans and Wells (1979a). Bacteria from a 50ml nutrient broth culture, incubated at  $37^{\circ}C$  for 18h were harvested by centrifugation at 1000g for 15 min in a bench centrifuge. The bacteria were resuspended in 10ml of PBS, a series of ten fold dilutions made and a viable count was performed by the method of Miles et al (1938). The undiluted suspensions and required dilutions were incorporated into a 5% suspension of hog gastric mucin, pH 7.2 (ICN Pharmaceuticals Inc, Plainview, New York, USA.), at a ratio of one part bacterial suspension to four parts mucin. Groups of 5 mice were inoculated i.p. with 0.5ml of each dilution and the number of deaths per group recorded up to 48h after challenge. The  $LD_{50}$  was calculated by the method of Reed and Muench (1938).

Mouse model of P.haemolytica infection: The model described by Evans and Wells (1979a) was adopted. Groups of ten mice were inoculated i.p. with at least 2  $LD_{50}$ s of the P.haemolytica isolate required in hog gastric mucin. After 6h the mice were killed by cervical dislocation and the livers removed aseptically. These were then individually macerated with 9ml of peptone water (Oxoid) in a Colworth stomacher (Seward, London). Serial ten-fold dilutions in peptone water were plated out for counting of viable cells by the method of Miles et al (1938). The mean viable count and standard deviation for each group was calculated and expressed as  $\log_{10}$  cfu.

Vaccines: Aqueous suspensions of the prepared antigens were either emulsified in equal volumes of complete Freund's Adjuvant(CFA) or absorbed onto 0.2% alhydrogel (Miles Laboratories Ltd,Stoke Poges,Slough)Antigen,water and Alhydrogel (5:2:4) were mixed and allowed to precipitate.This preparation was then emulsified in an equal volume of Bayol (Esso ) containing 10% Arlacel A (Sigma).

Vaccination and challenge of mice: Ten mice were each given two doses of 0.1ml of vaccine s.c. with 14 days between the two inoculations.Fourteen days after the second inoculation these mice and a group of uninoculated control mice were challenged i.p. as described in the mouse infection model.After 6h the mice were killed and viable counts performed on whole liver suspensions.The counts from vaccinated and control animals were statistically analysed using the Mann-Whitney Ranking Test (Snedecor and Cochran,1967).In early experiments a group of ten uninoculated control mice were killed immediately after challenge and the liver counts obtained were used as a base line count for the challenge.However,as the results of a number of experiments indicated that this value varied very little from  $1 \times 10^4$  this group was ommitted from later experiments.

Specific pathogen free (SPF) lamb model for P.haemolytica infection: The method used was that described by Sharp et al (1978).Four week old SPF lambs were allocated randomly into two groups of four which were housed in separate isolation rooms.Nasal swabs taken before challenge did not contain P.haemolytica.The lambs were infected with 8ml intratracheally

and 2ml intranasally of a suspension of parainfluenza virus type 3 (PI3) strain 92/425 ( $10^{6.75}$  TCID<sub>50</sub> per 0.2ml). Seven days later one group of lambs was exposed for 15min to an aerosol which contained  $4.6 \times 10^4$  cfu l<sup>-1</sup> of P.haemolytica isolate UT18 (IHA negative serogroup II). The second group was similarly infected with an aerosol of isolate UT2 (IHA negative serogroup V) at a concentration of  $7.0 \times 10^4$  cfu l<sup>-1</sup>. The lambs were examined clinically and at autopsy by the methods described by Gilmour et al (1982).

## RESULTS AND DISCUSSION

### SECTION 1.

#### The identification and isolation of a protective antigen from *P.haemolytica* serotype A1.

The antigens responsible for conferring protection against certain A biotype serotypes of *P.haemolytica* are known to be contained in extracts prepared by sodium salicylate treatment of cells from these serotypes (Gilmour et al, 1979 and 1983). These extracts are presumed to contain surface components and they include the serotype - specific antigen as sheep vaccinated with this extract in adjuvant produce high levels of antibody against the serotype antigen (Wells et al 1979). In addition, Gilmour et al (1979) have shown that protection is conferred on lambs vaccinated with SSE from serotypes A1, A2 and A6 when challenged with A1 and A6 but these lambs are susceptible to challenge with A9, a serotype not included in the vaccine. Thus the immunity is considered to be serotype-specific.

Identification of the specific antigen or antigens involved in protection would help in understanding the immunology of the disease. It should be possible by means of a range of different purification procedures and with the help of immunochemical techniques to identify and isolate the relevant antigens.

SECTION 1.1The Immunochemical analyses of extracts of *P.haemolytica*.Al cells.

The protein and carbohydrate compositions of SSE and EDTA extract, saline extract and PWE are shown in Table 1.1. SSE, EDTA and saline preparations were similar in composition with their protein to carbohydrate ratio ranging from 5.01 to 9.65 while the ratio for the PWE was substantially different at 0.31.

Crossed immunoelectrophoresis (CIE). The antigenic profiles obtained for each preparation run against antiserum to Al whole cell raised in rabbits are shown in Figure 1a-d. SSE (1.1a) gave one major peak which is almost a double peak with a strong cathodic line (close to origin) and a tapering anodic line. The EDTA preparation (1.1b) shows at least three and perhaps four different antigens: i) A fast running antigen producing a weak staining precipitin arc, ii) one antigen producing a strong staining precipitin, iii) one antigen producing a weak staining double precipitin arc close to the origin. The saline extract (1.1c) produced one very strongly staining complete precipitin arc while PWE reaction (1.1d) shows a strongly staining, large peaked precipitin arc running in front of a very weak staining double humped arc which is very close to the origin. The profiles obtained when PWE antigen was run in tandem with the other preparations are shown in Fig. 1.2. When run with SSE (1.2a) the single peak of the SSE fused with the fast running strongly staining precipitate of PWE, demonstrating that both antigens are identical. Slow moving antigen of PWE was



unaffected. The profile obtained with EDTA, extract and PWE, with the latter in the leading well in the tandem, is shown in Fig. 1.2b. The fusion of the two heavy staining peaks indicated their identity but the fast moving antigen of EDTA i, is unaffected. The EDTA iii antigen is identifiable by its double-humped precipitation but the slow moving precipitin arc of the PWE has disappeared. It is possible that these two antigens have fused resulting in one double humped precipitin arc. The tandem run of PWE with the saline extract is shown in Figure 1.2c. The main precipitin arcs of both preparations fused to confirm identity while the minor slow moving precipitin arc of PWE is seen directly below the saline precipitin arc.

The PWE antigen was also run in gels containing an intermediate gel loaded with antiserum against individual heterologous serotypes.

The results of the gels run with antiserum to A2, A6, A7 and A9 respectively are shown in Fig 1.3. The control gel did not have antiserum in the intermediate gel and the PWE antigen ran through this to precipitate in the main gel which contained homologous antiserum to A1 (Figure 1.3a). When the intermediate gel did contain antiserum to individual heterologous serotypes then the slow moving antigen close to the origin precipitated in the intermediate gel. The faster moving heavy staining peak was unimpaired and precipitated in the main gel.

TABLE 1.1

The protein and carbohydrate composition of extracts of P.haemolytica A1. (expressed as a percentage of total dry weight)

PREPARATION	PROTEIN	CARBOHYDRATE	PROTEIN/CARBOHYDRATE RATIO
SSE	57.9	6.0	9.65
EDTA extract	36.1	7.2	5.01
saline extract	45.8	6.1	7.51
PWE	6.3	20.1	0.31

**Figure 1.1**

Crossed immunoelectrophoresis of a)Al SSE (40ug) b)Al EDTA extract (40ug) c)Al saline extract (40ug) and d)Al PWE (10ug) run against rabbit antiserum to Al whole cells. The origins for these and all subsequent gels are located at the bottom right hand side.

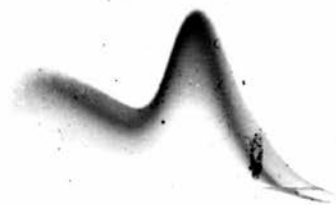
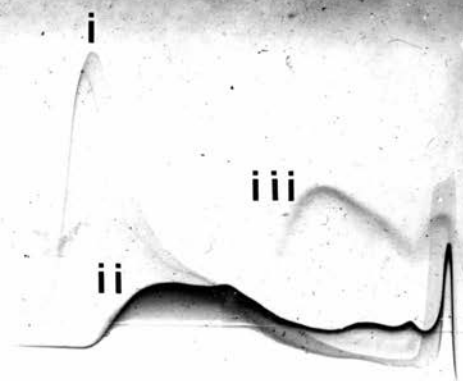
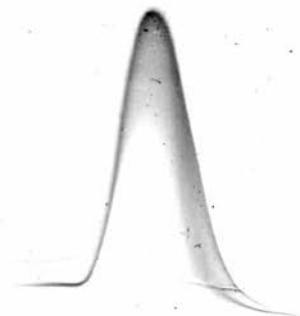
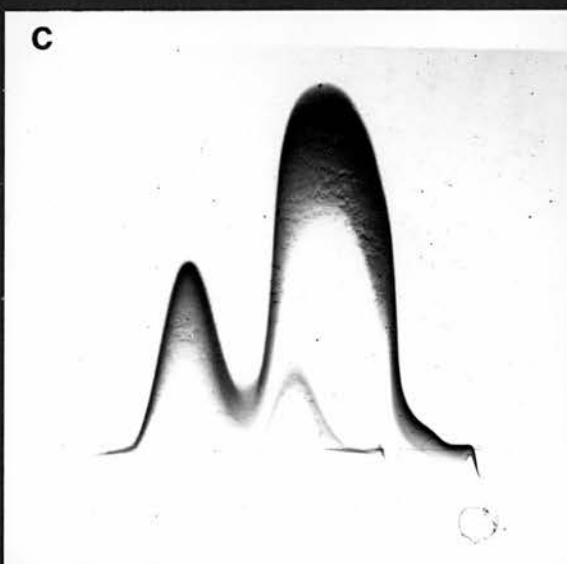
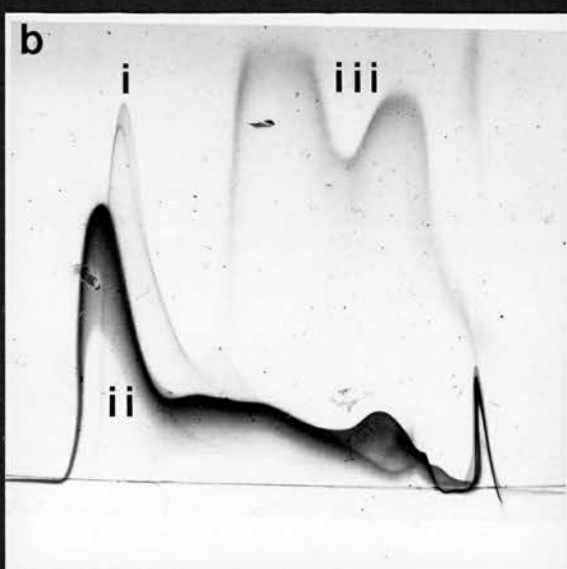
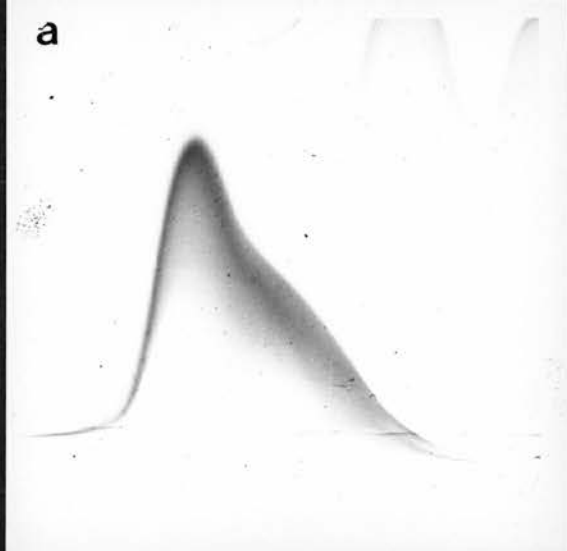
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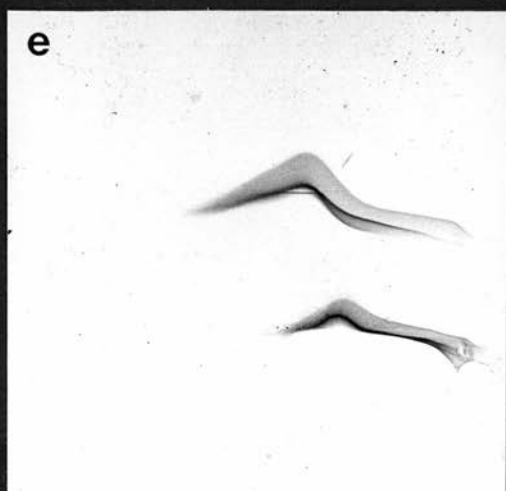
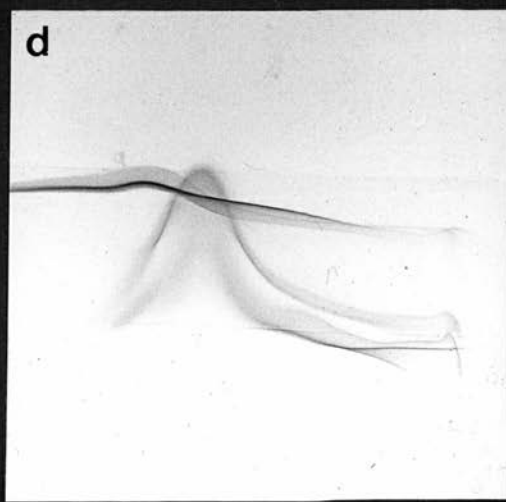
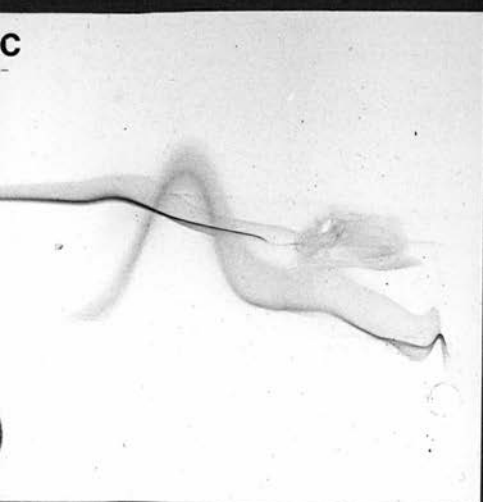
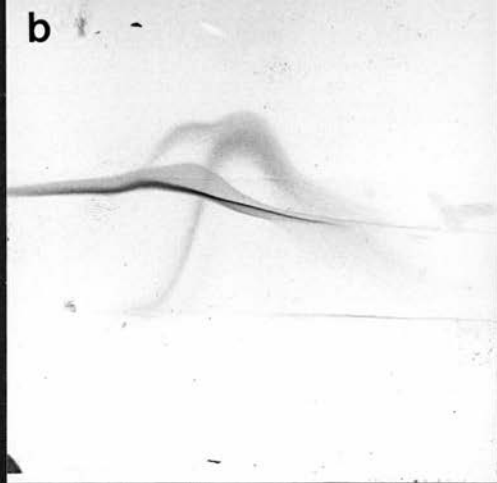
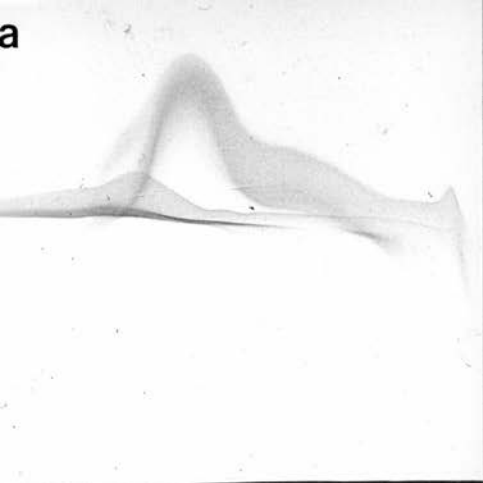
Figure 1.2

Crossed immunoelectrophoresis of Al PWE (10ug) when run in tandem with a)Al SSE (40ug), b)Al EDTA (40ug) and c)Al saline extract (40ug), against 130ul of rabbit antiserum to Al whole cells.



**Figure 1.3**

Crossed immunoelectrophoresis of A1 PWE (10ug) run against 100ul of rabbit antiserum to A1 whole cells with the incorporation of an intermediate gel containing a)no antiserum or 30ul of rabbit antiserum to b)A2, c)A6, d)A7 and e)A9.





## SECTION 1.2

### The immunogenicity of extracts of *P.haemolytica* Al cells

The immunogenicity of AlSSE in mice has been described by Evans and Wells (1979) and this model offers a means of assessing the protective capacity of the preparations.

Mice, vaccinated with AlSSE, EDTA extract, saline extract, PWE and heat killed organisms (HKO) all at a concentration of 0.58 ug ml<sup>-1</sup> dry weight were challenged with *P.haemolytica* Al as described in Materials and Methods.

The results are summarised in Table 1.2. All mice vaccinated with extracts or HKO had significantly lower liver counts than did the unvaccinated control group. Only in the control group did the bacteria multiply as shown by increased numbers of cfu after 6h compared to these obtained immediately after challenge. (4.6 to 6.1 (Log<sub>10</sub> values)). There is some indication that the degree of protection conferred by the different preparations, although not significant, is not the same, as the mean liver counts vary from 3.1±0.8 for the saline extract to 3.96±1.2 for PWE.

The results from section 1.1 indicated that a serotype-specific antigen was present in all the preparations and that PWE was apparently the simplest chemically and antigenically. This experiment showed that PWE can confer protection in mice and therefore that the protective antigen is present in this extract. Analysis of this extract would seem to be the most likely to provide information on the nature of the protective antigen.

TABLE 1.2

Colony forming units of P.haemolytica A1 in the liver of control mice and mice vaccinated with P.haemolytica A1 antigens after challenge.

VACCINE	CHALLENGE DOSE <sup>1</sup>	COUNT <sup>1</sup> IN LIVER AFTER		p <sup>2</sup>
		0h	6h	
SSE	6.32	4.6±0.5	3.50±1.0	<0.001
EDTA extract	6.32	4.6±0.5	3.30±1.0	<0.001
Saline extract	6.32	4.6±0.5	3.10±0.8	<0.001
PWE	6.32	4.6±0.5	3.96±1.2	<0.001
HKO	6.32	4.6±0.5	3.60±0.6	<0.001
Control	6.32	4.6±0.5	6.10±0.3	

1. Count expressed as Log<sub>10</sub> values

2. When compared with the control group count in the Mann-Whitney Ranking Test

### SECTION 1.3

The effect on the antigenicity of Al PWE when treated  
with sodium periodate, proteinase K or heat.

The antigen which confers protection on mice against challenge with P.haemolytica Al is present in PWE and this extract has been shown to contain protein and carbohydrate in the ratio 0.31 to 1. Treatment of PWE with reagents to destroy either the protein or carbohydrate components and the challenge of mice vaccinated with these treated PWE's might indicate which component is important in stimulating immunity.

Al PWE was prepared and aliquots treated with sodium periodate, proteinase K or heated at 115°C for 15 minutes as described in Materials and Methods. Untreated PWE and PWE treated as described above were incorporated into vaccines with FCA to a final concentration of 375ug ml<sup>-1</sup> of vaccine. The vaccination and challenge of mice has already been described.

The antigenicity of PWE before and after treatment as measured by the IHA test is shown in Table 1.3. Only treatment with periodate removed the IHA titre. The other treatments had no effect.

The gel patterns obtained when the four samples were run in CIE against homologous antiserum are shown in figure 1.4 (a-d). The samples were run through an intermediate gel containing antiserum against A9 whole cells (Fig 1.4 e-h). The patterns are approximately similar for all four samples with a strong cathodic line and an anodic line fading and tapering. However, the periodate treated sample is different from the other three

when run through the heterologous antiserum as the cross-reacting antigen is not present (Fig 1.4g).

The effectiveness of the samples in stimulating protection was determined in the mouse model . The results are shown in Table 1.4.

The periodate treated PWE did not give protection while the other three preparations did. The order of protective efficacy, as indicated by the Mann-Whitney Ranking Test was untreated PWE > proteinase K treated PWE > heat treated PWE.

It can be concluded from this experiment that the IHA antigen and the protective antigen are periodate sensitive and therefore a carbohydrate component of PWE is an important antigen in the protection of mice against challenge with P.haemolytica.

Table 1.3

IHA titre of Al PWE before and after treatment with sodium periodate, proteinase K or heat.

TREATMENT	IHA TITRE
None	100
Proteinase K	100
Sodium periodate	2
Heat (115°C)	100

Table 1.4

Colony forming units of P.haemolytica in the livers of mice vaccinated with Al PWE treated with proteinase K, sodium periodate or heat after challenge with P.haemolytica Al.

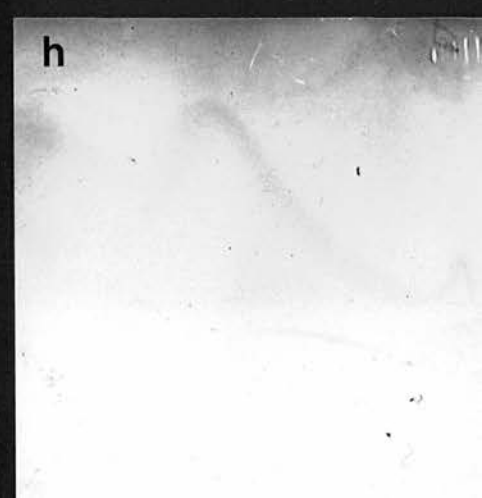
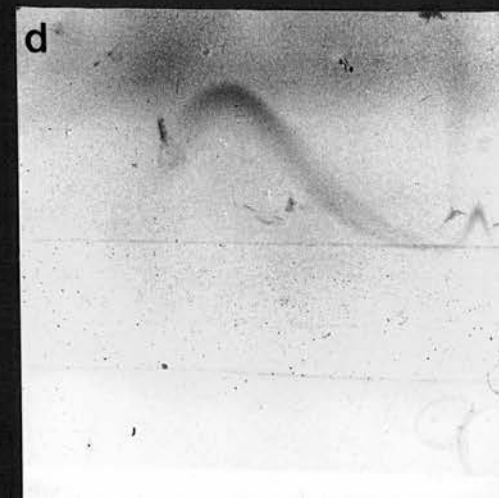
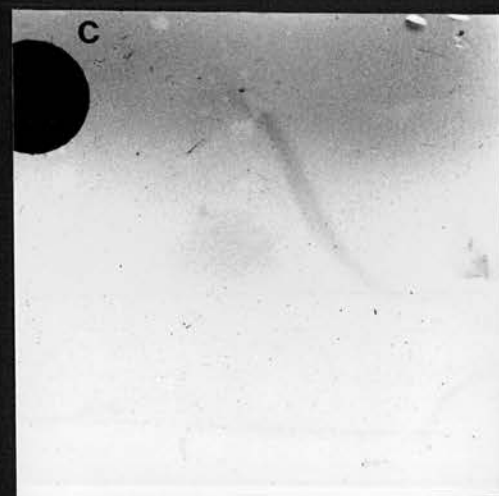
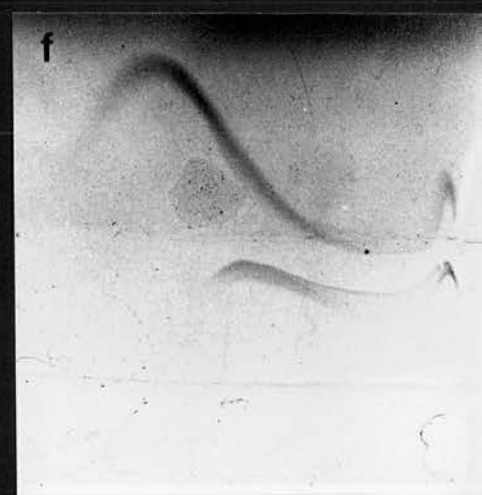
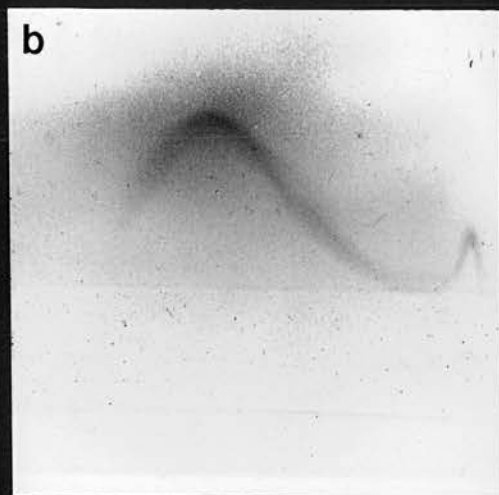
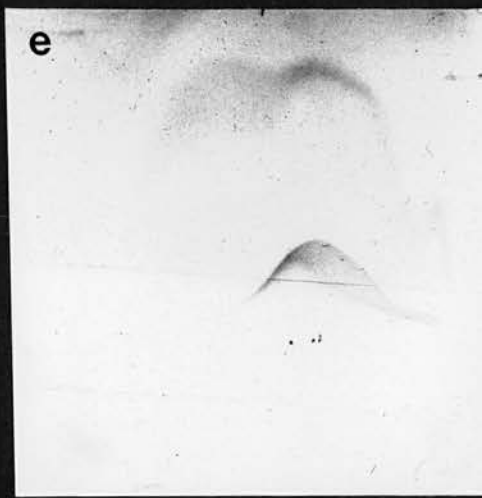
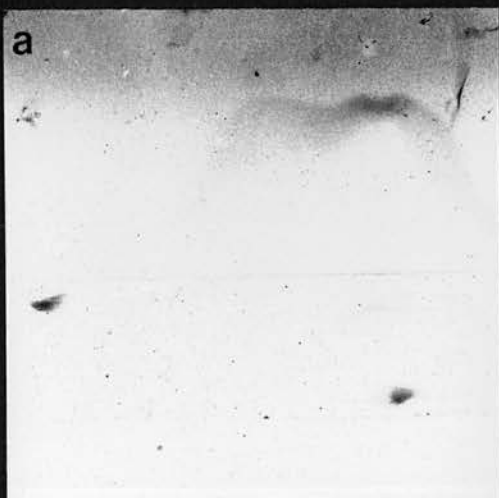
VACCINE	TREATMENT	LIVER COUNT 6h p.i. (Log <sub>10</sub> cfu)	P <sup>1</sup>
PWE	None	2.76±0.2	.001
PWE	Proteinase K	3.19±1.0	.005
PWE	Sodium periodate	3.59±1.1	NS
PWE	Heat	3.54±1.4	.001
None	-	4.50±1.3	

1. P= significance when compared with the control group count in the Mann-Whitney Ranking Test.

NS= not significant

Figure 1.4

Crossed immunoelectrophoresis of A1 PWE preparations (10ug) run against 130ul of rabbit raised against A1 whole cells; a)untreated; b)proteinase K treated; c)sodium periodate treated and d)heat (115°C) treated. The same samples were also run with an intermediate gel containing 30ul rabbit antiserum to A9 whole cells; e)untreated; f)proteinase K treated; g)sodium periodate treated and h)heat treated.



### Discussion

SSE of P.haemolytica A1 has been shown by Gilmour et al (1979, 1983) to protect against challenge with P.haemolytica A1 in sheep but no work has been carried out on the antigens responsible for this protection. The results of this preliminary investigation into the antigenicity of P.haemolytica A1 showed that the SSE, EDTA saline extracts and PWE contained a serotype-specific antigen.

Crossed immunoelectrophoresis examinations of the structure and antigenic composition of other bacteria such as E.coli (Smyth et al, 1978), P.multocida (Bhasin and Lapointe-Shaw, 1980) N.meningitidis (Hoff and Frasch, 1979) and Bacteroides vulgatus (Poxton and Ip, 1981) have indicated the usefulness of CIE in identifying and characterising antigens. However, it has highlighted the antigenic complexity of the bacterial cell. The results of the CIE on extracts of P.haemolytica cells show the presence of a distinct serotype antigen in all preparations. The lack of reaction with heterologous antisera is strong evidence that this antigen is the serotype antigen. The number of different antigens present in the extracts was low compared to that reported for E.coli (Smyth et al, 1978) in which at least 25 immunoprecipitates were observed in outer membrane preparations and for the related organism, P.multocida in which 19 envelope antigens were observed (Bhasin and Lapointe-Shaw, 1980). The paucity of reactions with P.haemolytica extracts may be due to the immunodominance of the serotype antigen which is obscuring the reactions of weaker antigens.



A reason for this observation may lie in the few passages that this strain has been subjected to in the laboratory from the time it was isolated from a field case of pneumonic pasteurellosis. Poxton (personal communication) has found that fresh clinical isolates of Gram-positive bacteria have a simpler antigenic profile than the same organisms after subsequent laboratory passage. Costerton et al (1981) has suggested that this may occur because the glycocalyx (a polysaccharide layer external to the LPS) present in vivo is lost on passage in vitro as the media would preferentially allow the growth of mutants not producing an energy demanding glycocalyx. Another explanation of this may be that the sodium salicylate denatures the other antigens. However, very little is known of the action of sodium salicylate on the bacterial cells and the only other bacterial cells treated with this salt were those of Yersinia (Pasteurella) pestis (Amies, 1952). Amies suggested that it acted to disrupt the outer layers.

The result of section 1.2 showed that the antigen (or antigens) responsible for mouse protection are present in all the extracts. PWE was shown to be predominantly polysaccharide in nature compared to the protein-rich composition of EDTA, SSE and saline preparations and the importance of polysaccharide antigen was demonstrated by the abrogation of protective immunity after treatment of PWE with sodium periodate. This finding together with the coincidental loss of IHA activity of the sample indicated that the antigens responsible for protection and serotype specificities are related and may be identical.

It is interesting that PWE was probably the least effective immunogen but a possible explanation of this may lie in its predominantly polysaccharide composition. Karch and Nixdorff (1981) have shown that antibody-producing cells of mice gave an increased response to the LPS of Proteus mirabilis when it was combined with an outer membrane compared to that of LPS alone. A similar finding was reported for E.coli by Ahlstedt and Lindholm (1977) who found that a small molecular weight protein was associated with the immune response to LPS. Removal of the protein reduced the response to the LPS antigen while coinjection of LPS with the protein, either linked or separate, restored the level of the response. It may be that the LPS of P.haemolytica also requires some close association with proteins to enhance its protective immunogenicity. The protein could be acting as an antigenic carrier and the response to the LPS connected with a response of T-helper cells to the protein. When T-cells recognise and respond to the carrier determinants they help B-lymphocytes specific for the hapten (LPS), to develop into antibody forming cells (Roitt, 1980).

The results of previous studies present conflicting evidence as to the antigens important in protection. Biberstein and Thompson (1965) found that in mice vaccinated with whole cells, the serotype antigen was the major influence on protection with so-called somatic antigens playing a lesser role. However, Knight et al (1969), also working with mice presented contradictory results indicating that strains unrelated in IHA serotype or somatic antigenicity cross protected. More recently Tadayon and Lauerman (1981) have reported that an extract of

P.haemolytica, prepared by treatment of the cells with potassium thiocyanate (KSCN) was effective in protecting mice and hamsters against homologous challenge whereas an LPS preparation did not. However, neither the serotype used nor the challenge method was detailed so it is difficult to gauge the significance of their findings.

Phenol-water treatment is generally regarded as a method for extracting LPS from Gram-negative bacteria and it is therefore likely that it has the same effect on P.haemolytica. Two distinct antigens are present in PWE when visualised in CIE, one a serotype-specific antigen producing a strong precipitin arc the other producing a weak precipitin line in the form of a double humped peak close to the origin, was common to all serotypes tested. The main antigen seems to comprise two closely spaced lines, which is similar to the pattern obtained for N.meningitidis LPS by Hoff and Frasch (1979) who suggested that microheterogeneities in the molecule might be responsible.

SECTION 2.The characterisation of the protective antigen of *P.haemolytica*A1.

Components of both a phenol-water or sodium salicylate extract of cells of *P.haemolytica* have been shown to protect against challenge with the homologous serotype (Section 1.2). The hot phenol water extraction procedure used to produce the PWE is generally recognised as a preparative procedure for Gram-negative LPS (Westphal et al, 1952) and as the results of Section 1 implied that a polysaccharide antigen was important in protection, then the LPS is possibly the protective antigen.

A combination of chromatographical and traditional chemical analytical techniques should help to elucidate the composition of the *P.haemolytica* A1 polysaccharide antigen.

SECTION 2.1The analyses of the polysaccharide antigens of *P.haemolytica*Al.

Lipopolysaccharide preparations of many Gram-negative bacteria can be hydrolysed with 1% acetic acid to break the link between lipid A and the core polysaccharide/ antigenic "O" chain and the components can be isolated by centrifugation and chromatography (Schmidt et al, 1969; Fensom and Meadow, 1970). The "O" antigen obtained by this method can then be fractionated and analysed. This section describes the the isolation, fractionation and analyses of the presumptive polysaccharide of *P.haemolytica* Al PWE. PWE (10 mg), hydrolysed with 1% acetic acid as described in Materials and Methods, and untreated PWE were chromatographed on a Sephadex G50 column. The fractions were analysed for phosphate, protein and sugar content. They were also tested for antigenicity by fused-rocket immunoelectrophoresis (FRIE). The sandwich enzyme-linked immunosorbent assay (ELISA) described in Section 3 was also used to measure antigenicity in column fractions. Sheep antiserum raised against Al SSE was used at a dilution of 1 in 500 as the detecting antiserum. PWE was analysed for heptose, KDO, uronic acids, fatty acids and N-acetyl neuraminic acid by the methods given in Materials and Methods.

Yield of PWE: From 2.8g dry weight of cells, 44mg of extract were obtained (i.e. 1.6%).

Chemical analysis: The total sugar, phosphate, heptose, protein, KDO and fatty acid content of PWE is shown in Table 2.1. No uronic acids or N-acetyl neuraminic acid were detected.

Paper chromatography: The PWE showed spots corresponding to standards of glucose, galactose, glucosamine, galactosamine and ribose. There were no spots corresponding to glucuronic acid, mannose, fucose, rhamnose or xylose.

Column chromatography: The elution patterns obtained when samples of untreated or partially hydrolysed PWE were chromatographed on Sephadex G-50 and the fractions were assayed for total reducing sugar and free or total phosphate are shown in Figure 2.1. When assayed for reducing sugar untreated PWE was shown to elute from the column in fraction numbers (FN) 15-29 while partially hydrolysed PWE eluted at three distinct peaks; i) FN 15-23 ii) 31-40 and iii) 41-45. The second peak (ii) contained most material. The profile of phosphate elution for partially hydrolysed PWE is approximately similar to that obtained for reducing sugars and shows three peaks; 1) FN 17-25 2) 29-35 3) 41-45.

The sephadex G50 fractions obtained for untreated and partially hydrolysed PWE were run in FRIE. The antigen antibody reactions obtained are shown in Figure 2.2. The early fractions, FN17-29, recovered from the separation of untreated PWE had antigenic activity (Fig 2.2a and b) and these correspond to the fractions showing measurable sugar content as demonstrated in Figure 2.1. The early fractions of partially hydrolysed PWE, FN 16,17 had slight antigenic activity (Fig 2.2c and d) which correspond to the first eluted peaks seen for partially hydrolysed PWE in Fig 2.1. However, the rockets as shown in Figure 2.2c are very weakly stained when compared to the reference sample at the left side of the gel. Only a small

proportion of the total sugar content of partially hydrolysed PWE is found in these early fractions. The greater proportion is found in the later fractions which do not show any antigenic activity. As partial acid hydrolysis may have affected the precipitating properties of the antigen a "sandwich" ELISA was also used to measure the antigenic content of the sample. The results are summarised in Table 2.2. Only two pools, FN11-16 and 17-23 showed high antigenicity.

The results of reducing sugar assay, FRIE and ELISA for untreated PWE and partially hydrolysed PWE are summarised in Table 2.3.

Gas chromatography analysis: The following samples were examined: untreated PWE, partially hydrolysed PWE, Lipid A and pooled fractions from the major peak ii, obtained in the G50 separation of partially hydrolysed PWE. Traces showing the profiles of alditol acetate derivations of hydrolysed samples are reproduced in Figure 2.3. The sugars identified in each sample and their molar ratios are tabulated in Table 2.4. The two peaks falling between the hexose and amino sugar derivatives were provisionally identified as heptose derivatives. A sample from each of the two peaks was collected by Dr. D.L. Mould at Moredun Research Institute and was subjected to analysis by mass spectrometry (MS) at the Rowett Research Institute by Dr. J.A. Lomax. Both were identified as heptitol heptaacetates. PWE, and Lipid A contain exactly the same components but with some qualitative differences. Partially hydrolysed PWE and the major product of partial acid hydrolysis, peak ii, contained glucose, mannose, galactose and two heptoses.



From these results it can be proposed that the phenol water extract of P.haemolytica A1 is composed mainly of carbohydrate, a small proportion of lipid and an even smaller proportion of protein. The PWE is sensitive to acid hydrolysis and loses antigenicity after treatment. Two distinct peaks on G.C. analysis proved to be heptoses.

Table 2.1

Composition of A1 PWE (expressed as a percentage of total dry weight).

---

Phosphorus	1
Protein	6.3
Carbohydrate	14.8
Fatty acid	10.8
Heptose	6.6
KDO	0.6

---

Figure 2.1

The reducing sugar and phosphorus content (A490 and A820 respectively) of fractions obtained after separation of untreated Al PWE and partially hydrolysed Al PWE on a Sephadex G50 column.

●—● = Untreated PWE

○—○ = Partially hydrolysed PWE

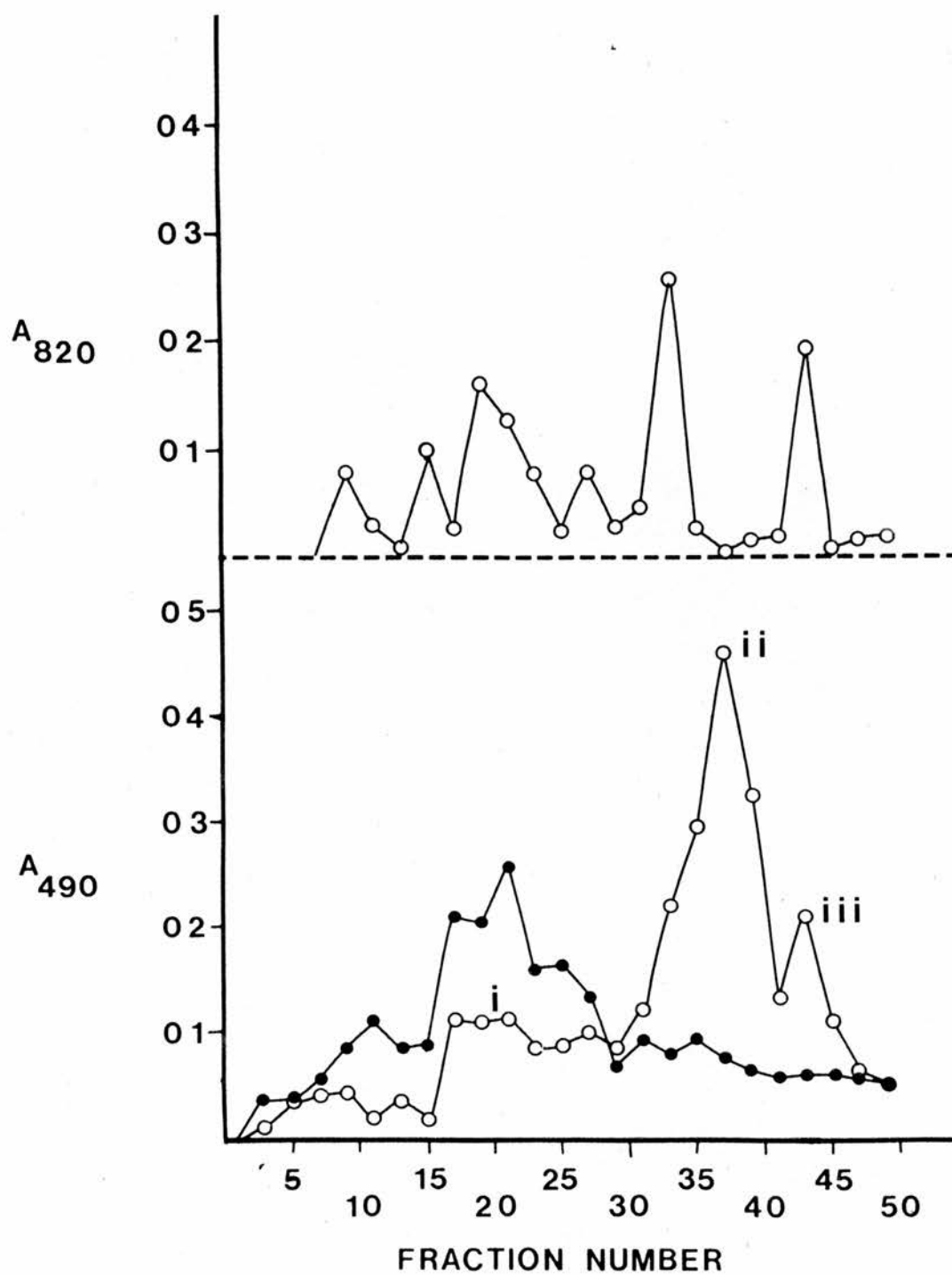


Figure 2.2

Fused rocket immunoelectrophoresis of Sephadex G50 column fractions of a) untreated PWE, fractions 13-29; b) untreated PWE, fractions 31-39, c) partially hydrolysed PWE, fractions 11-28, d) partially hydrolysed PWE, fractions 29-49, when run in 1% agarose containing rabbit antiserum to A1 whole cells. A reference sample of PWE (40ug) was run in each gel (X)

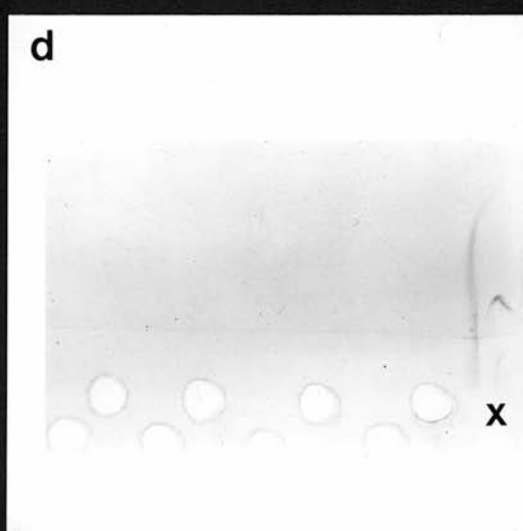
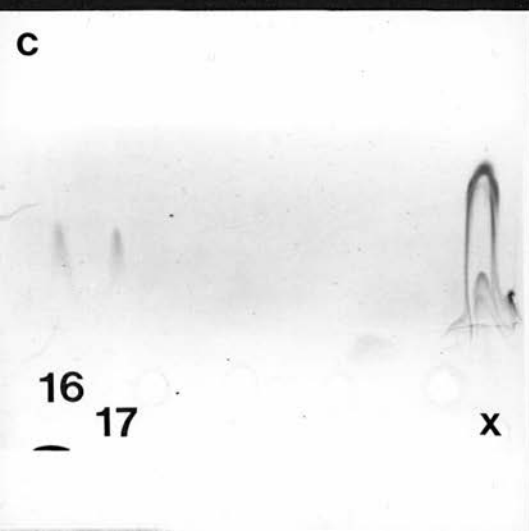
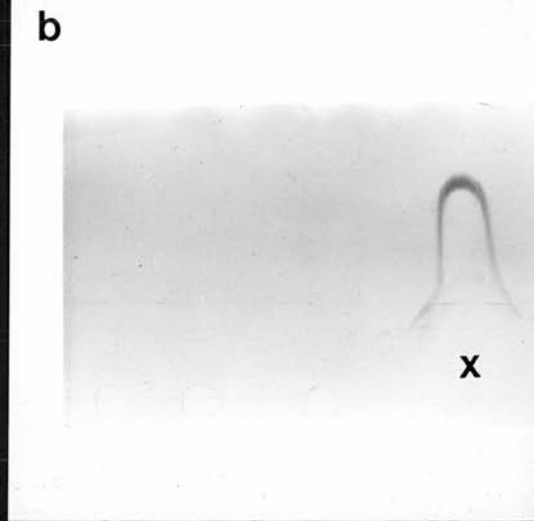
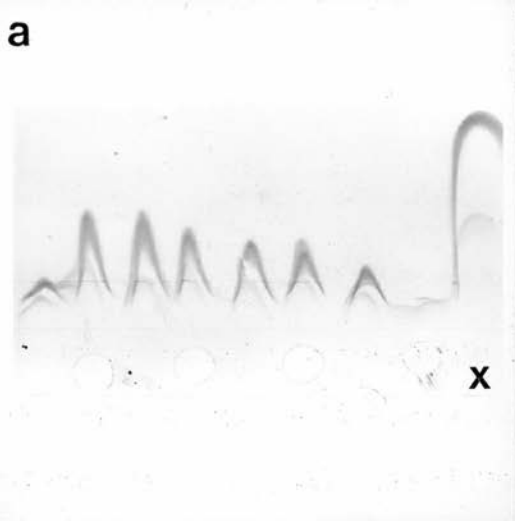


Table 2.2

The antigenicity in sandwich ELISA of Sephadex G50 column fractions (A405 values given).

FRACTION POOL	ABSORBANCE READINGS (A405) AT THE FOLLOWING DILUTIONS OF ANTIGENS		
	10	100	1000
1 - 10	0	0	0
11- 16	0.9	0.56	0.1
17- 23	0.4	0.1	0.1
24- 35	0	0.12	0.08
36- 60	0	0	0
Untreated PWE	2.4	1.8	0.98

Table 2.3

Summary of results of analyses on Sephadex G50 column fractions of untreated and partially hydrolysed PWE.

SAMPLE	FRACTION No.	ANTIGEN DETECTED BY		SUGAR CONTENT
		FRIE	ELISA	
Untreated PWE	1 - 14	-	ND	-
	15- 29	+	ND	High
	30- 34	-	ND	-
	40- 50	-	ND	-
Partially hydrolysed PWE	1 - 18	Trace	+	Trace
	19- 25	Trace	+	Trace
	26- 35	-	-	High
	36- 45	-	-	moderate
	46- 50	-	-	-

ND = Not done.

+ = present

- = not present



Figure 2.3

GC of alditol acetate derivatives of acid hydrolysates (2M HCl, 100°C 2h) of P.haemolytica Al PWE preparations on columns of OV225 for detection of sugars. GC conditions: N<sub>2</sub> 20ml min<sup>-1</sup> H<sub>2</sub> 20ml min<sup>-1</sup> and air 525ml min<sup>-1</sup>, temperature programmed between 190°C and 210°C at 2°C min<sup>-1</sup>.

a) untreated PWE; b) partially hydrolysed PWE c) pooled fractions of peak II; d) Lipid A. The sugars present are identified as follows: 1, mannose; 2, galactose; 3, glucose; 4, heptose 1; 5, heptose 2; 6, glucosamine; 7, galactosamine.

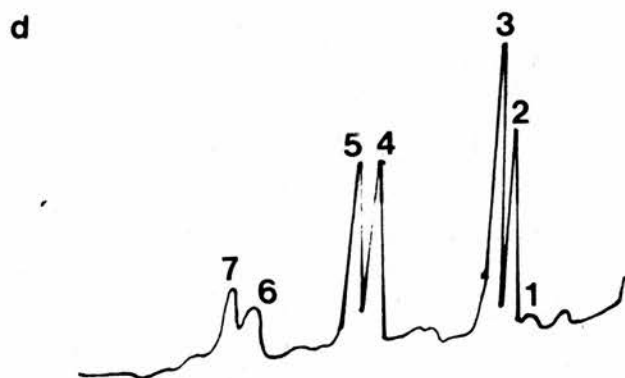
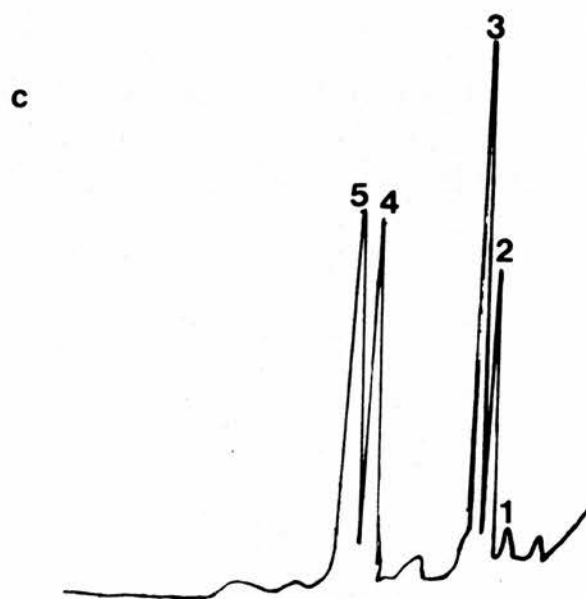
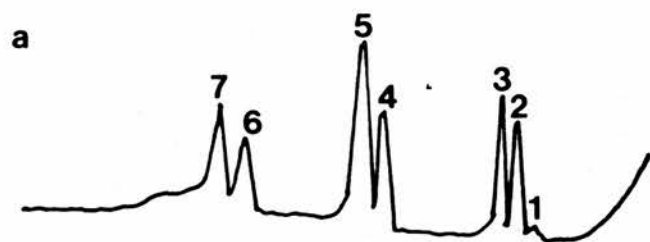


Table 2.4

The presence and molar ratios (sugar/glucose) of sugars in Al  
PWE samples as detected gas chromatography. The sugars are  
identified as follows: 1, mannose; 2, galactose; 3, glucose; 4,  
heptose 1; 5, heptose 2; 6, glucosamine; 7, galactosamine.

SAMPLE	SUGAR						
	1	2	3	4	5	6	7
PWE	0.08	0.83	1.0	0.83	1.3	0.6	0.83
Lipid A	0.04	0.8	1.0	0.7	0.6	0.2	0.23
Partially hydrolysed PWE	0.08	1.0	1.0	0.7	0.8	0	0
Peak ii	0.06	0.6	1.0	0.7	0.73	0	0

## SECTION 2.2

### The further characterisation of the PWE antigens of *P.haemolytica* A1.

The results in section 1.1 have demonstrated that more than one antigen existed within the PWE antigenic complex, as measured with CIE. These antigens are carbohydrate in nature and the evidence suggests a lipopolysaccharide composition. However, it has been reported that capsules are present on *P.haemolytica* (Biberstein and Thompson, 1965) and it may be that one or more of the antigens present in the PWE are capsular in nature.

Three different approaches were taken in attempting to identify the antigens; differential isolation of the antigens from the PWE by i)affinity chromatography, ii)elecrophoresis and iii)the removal of surface material likely to be capsular in nature before the cells were extracted with phenol water.

The methods have been described fully in Materials and Methods.

i)Affinity chromatography: The gels obtained when fractions of PWE from a CNBR sepharose 4B column with rabbit antisera to A9 bound to it were tested in FRIE against antiserum to A1 are shown in Figure 2.4. The eluted fractions (Fig 2.4a) still contained two antigens indicating that the cross-reacting antigen had not been removed. The desorbed fractions (Fig 2.4b) contained less antigen than the eluted fractions but again two antigens are clearly seen, especially in fraction 5. This suggested that the column was binding two antigens and not just

the cross-reacting antigen. The antigenicity of the fractions was assayed by the ELISA and the results are shown in Figure 2.5. The high A405 values shown by eluted fractions 2-4 against antiserum to P.haemolytica A9 indicates that the eluant still contained cross-reacting antigen.

ii) Electrophoretic separation: The supernate obtained after washing of the agarose gel and the residual agarose were run in rocket electrophoresis (Fig 2.6). A single antigen was precipitated from both samples when run against homologous rabbit antiserum whereas the reference preparation, PWE (5ug ml<sup>-1</sup>) showed that at least two distinct lines (Fig 2.6a). When the same samples were run in a gel containing an intermediate gel loaded with rabbit antiserum to A9 cells the supernate and agarose preparations showed only one antigen which precipitated in the main gel but the weaker staining antigen of the reference sample was precipitated in the intermediate gel indicating its cross-reactivity. The supernate and PWE were also tested in ELISA and the results are shown in Table 2.5. The absence of reactivity between the supernate and the antiserum to A9 (A405 < 0.1) compared to the reaction between A1 PWE and the same antiserum (A405=1.1) confirms the purity of this antigen.

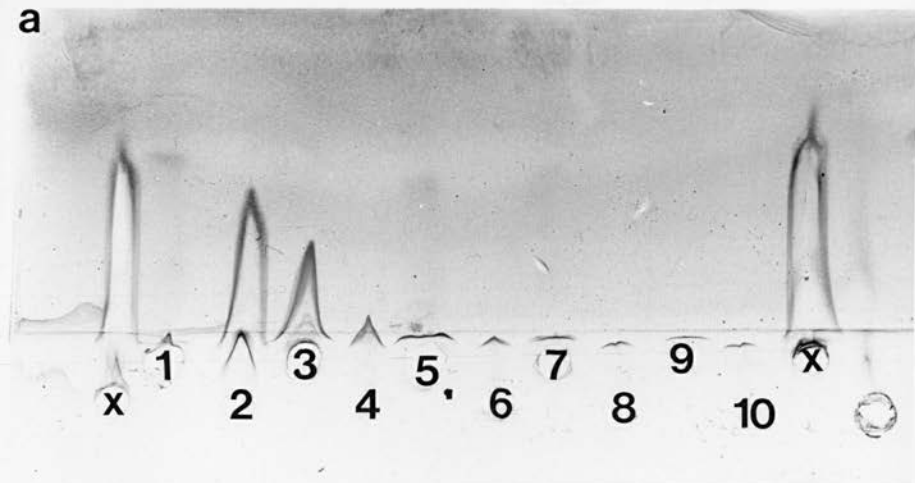
iii) Sequential treatment of A1 cells: Saline extracts and subsequent PWE of the same cells were tested in CIE as shown in Figure 2.7. In both preparations two antigens were present, one of which, the precipitin line nearest to the origin, was precipitated in the intermediate gel containing antiserum to A9. The antigenicity of both samples was also tested in ELISA (Fig 2.8). Both preparations had high antigenicity when reacted with

antiserum against A1 SSE with the PWE being more antigenic. When the same preparations were reacted with antiserum to A9 the A405 values were substantially lower but again the antigenicity of PWE was greater. However there does not seem to be any convincing evidence that pre-treatment of P.haemolytica A1 cells with saline before phenol water extraction removes a capsular layer.

Figure 2.4

Fused rocket immunoelectrophoresis of fractions obtained after affinity chromatography of A1 PWE on immunosorbent column containing rabbit antibody to A9. a) eluted fractions 11-10; b) desorbed fractions 1-10. A1 PWE (10ug) was included in each run as a positive control (X).

a



b

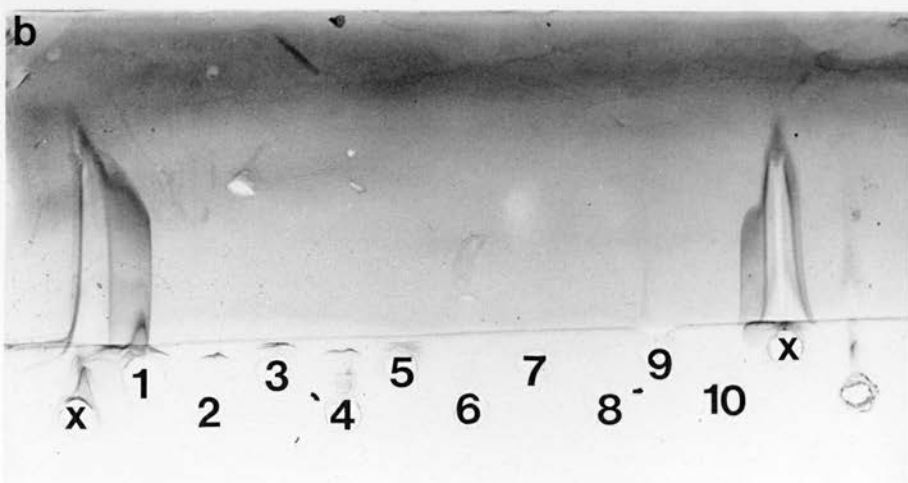






Figure 2.5

ELISA reactions of eluted (  ) and desorbed (  ) fractions obtained after affinity chromatography of A1 PWE on an immunosorbent column containing rabbit antibody to serotype A9 whole cells when tested against a) sheep antiserum to A1 whole cells and b) sheep antiserum to A9 whole cells.

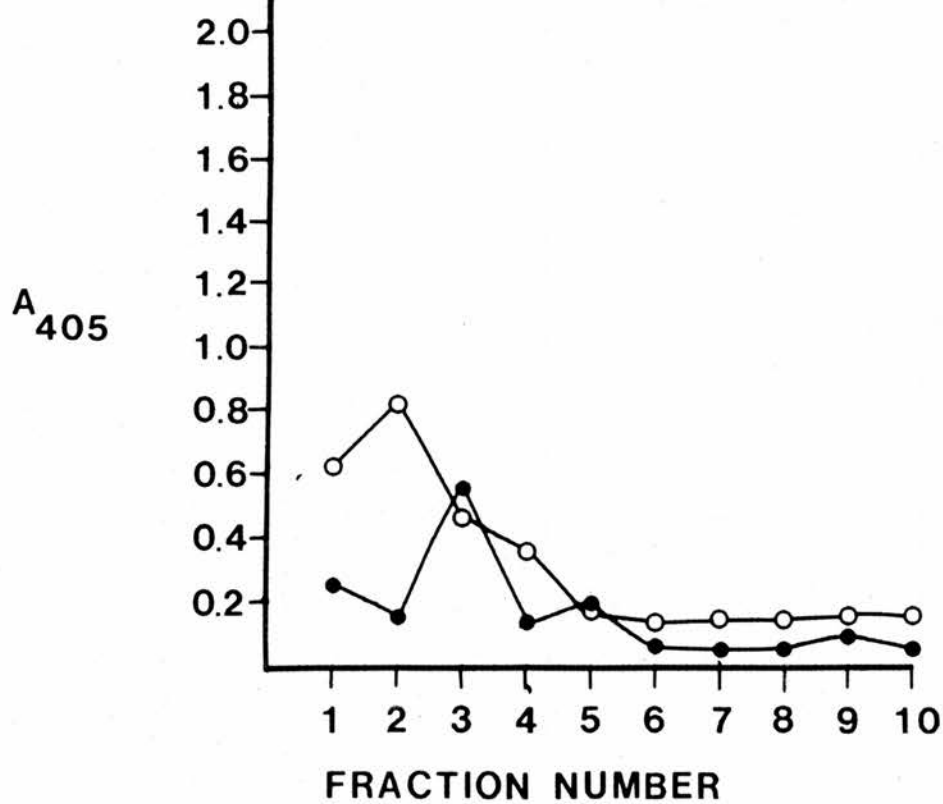
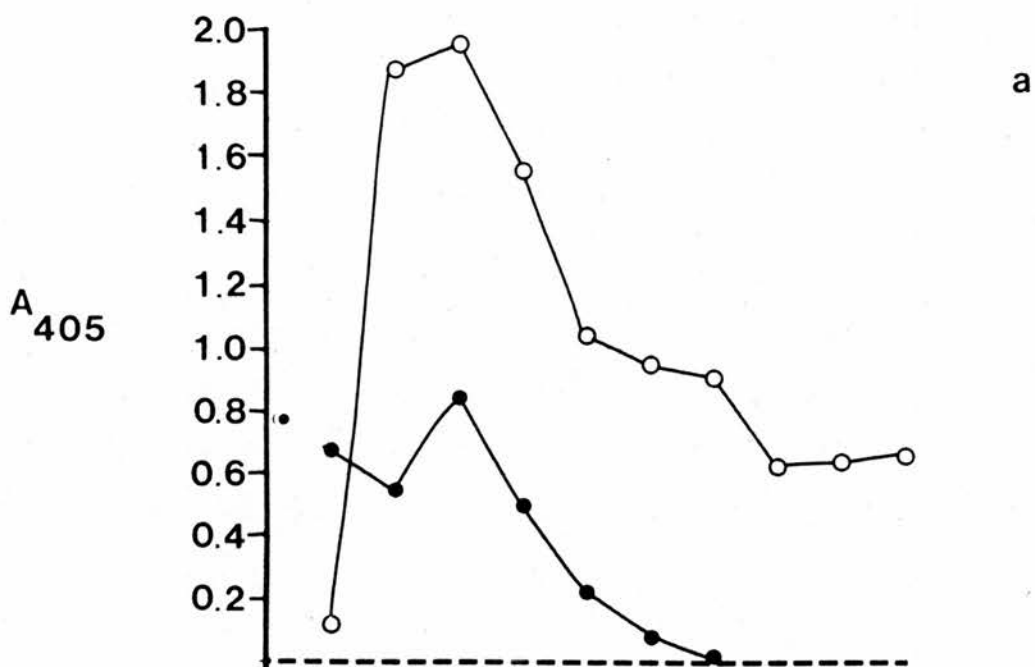


Figure 2.6

Fused rocket immunoelectrophoresis of supernate (1) residual agarose (2) and A1 PWE ( $5\text{mg ml}^{-1}$ ) (3) when run against a) rabbit antiserum to A1 whole cells and b) rabbit antiserum to A1 whole cells with an intermediate gel containing rabbit antiserum to A9 whole cells.

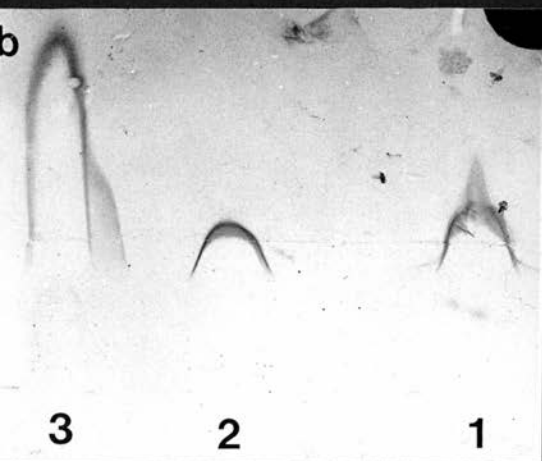
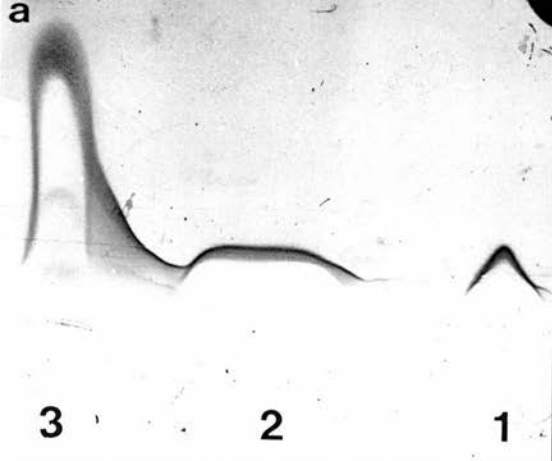


Table 2.5

Absorbance readings (A405) obtained from antigen isolated by electrophoresis from A1 PWE when assayed in a sandwich ELISA (For method see Materials and Methods).

SAMPLE	ANTISERUM	(A405) READING AT THE FOLLOWING DILUTIONS OF ANTIGEN				
		10	50	100	200	1000
Supernate	Anti A1	0.934	0.761	0.278	0.155	-
Supernate	Anti A9	0.09	0.08	0.06	0.07	-
A1 PWE	Anti A1	-	-	-	-	2.5
	Anti A9	-	-	-	-	1.1

- = not done

Figure 2.7

Crossed immunoelectrophoresis of a)PWE extract and b)PWE from A1 cells run against 130ul of rabbit antiserum to A1 whole cells. The samples were also run against an intermediate gel incorporating 30ul of antiserum to A9 whole cells c)saline extract d)PWE.

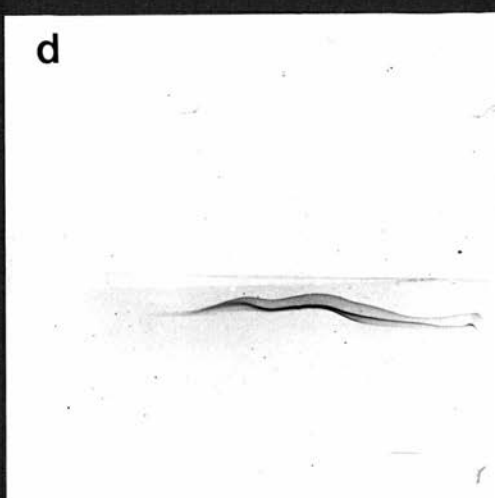
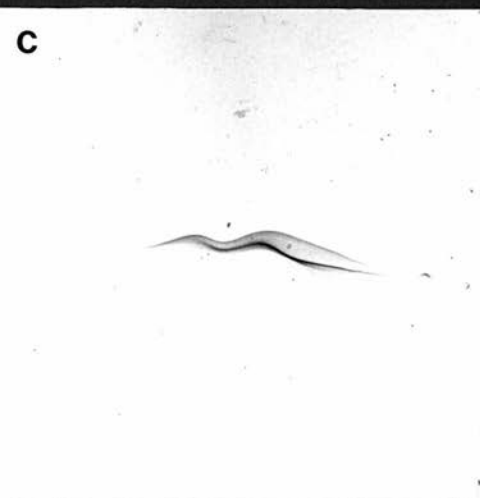
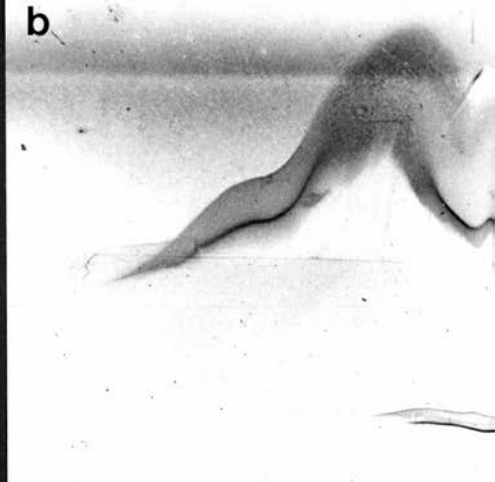
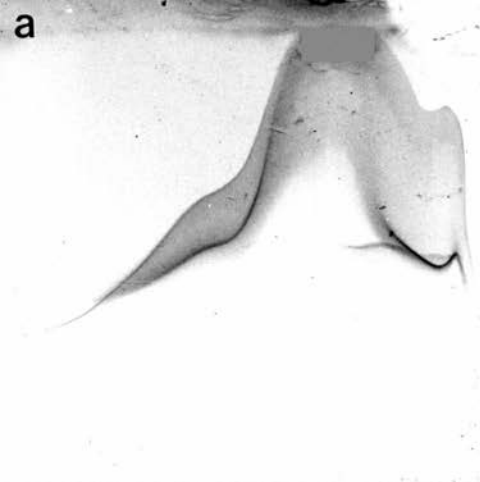


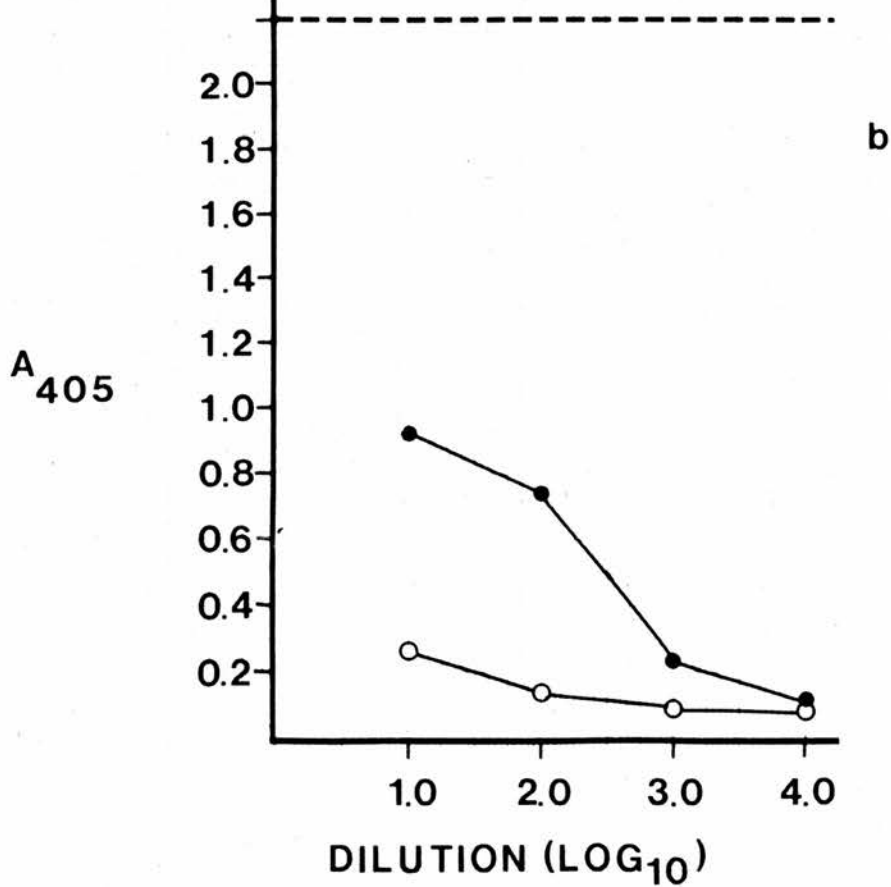
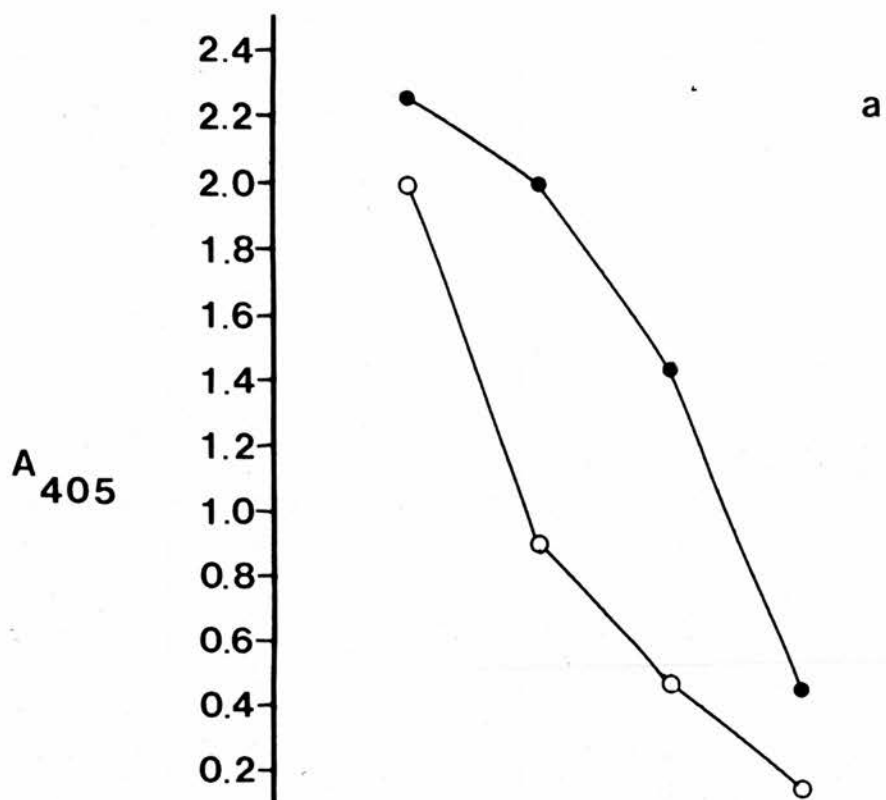
Figure 2.8

Titration of PWE and saline extract from galactose agar grown cells of P.haemolytica A1 by ELISA. These antigens were reacted with a) sheep antiserum to serotype A1 and b) sheep antiserum to serotype A9.

●—● = PWE

○—○ = Saline extract





## DISCUSSION

The PWE of P.haemolytica A1 is predominantly carbohydrate in composition with protein, fatty acids and phosphate associated with it. The presence of heptose and KDO is good evidence for assuming that lipopolysaccharide is present as these two molecules are regarded as markers for this important component of the outer membranes of the Gram-negative bacteria. The proportion of sugar in PWE is similar to the proportion of sugar in the LPS of other Gram-negative bacteria such as Vibrio cholerae (Raziuddin, 1980) and Yersinia pseudotuberculosis (Brubaker et al, 1973). However the amount of sugar present in the LPS can vary substantially especially if rough mutants are present (Poxton and Sutherland, 1976).

The molar ratios of the heptose and total sugars, (1:2.2, moles heptose/moles total sugar) as determined by GC, show that the heptoses constitute a major portion of the extract. The heptose/total sugar ratios in the LPS of some other Gram-negative bacteria are 1:2.4 for Yersinia pseudotuberculosis (Brubaker et al, 1973), 1:5 for Vibrio cholerae (Raziuddin, 1980) and 1:7.7 for Klebsiella pneumoniae (aerogenes) (Poxton and Sutherland, 1976).

As the proportion of heptose in A1 PWE is almost in this range it suggests that all the other sugars can be accounted for as part of the LPS making the presence of capsular polysaccharide unlikely. No pentoses, dideoxy sugars or uronic acids seem to form part of the PWE. However, the results also indicate that about 50% of the PWE could not be accounted for and it is possible that capsular components could be in this.

Partial acid hydrolysis of LPS from Gram-negative bacteria such as E.coli (Schmidt et al, 1969) Klebsiella pneumoniae (aerogenes) (Poxton and Sutherland, 1976), Vibrio cholerae (Raziuddin, 1980) and Pseudomonas aeruginosa (Fensom and Meadow, 1970) gave rise to fractions containing degraded polysaccharide (water soluble), lipid A (chloroform soluble) and interfacial material. When the degraded polysaccharide material is chromatographed on a Sephadex G50 column two main peaks are usually observed. The first peak, eluting just after the void volume, contains the "O" antigen chain while the second peak which is held back in the column corresponds with the core polysaccharide. The results obtained with the PWE of P.haemolytica A1 did not follow this pattern. Only one major peak was obtained after hydrolysis and the elution time correlated with the core containing peak. The lack of antigenicity in any of the fractions indicates that antigenicity is destroyed by the treatment and this finding is associated with the loss of amino sugars after partial acid hydrolysis. This suggests that the LPS of P.haemolytica is a rough type containing a core polysaccharide and perhaps a short "O" antigen chain. This chain probably contains the two amino sugars and is completely degraded after partial acid hydrolysis.

An interesting feature of PWE composition, as determined by GC, was the presence of two distinct heptoses which are clearly separated on the GC column but are indistinguishable when analysed by mass spectrometry. These two heptoses cannot be alpha and beta forms as these would run identically in the column and therefore must be distinct heptoses. However,

attempts to identify them by coinjection with known heptose standards have proved unsuccessful. The genus Pasteurella is closely related to the genus Yersinia and in this genus unusual heptoses have been identified (Wilkinson, 1977, Brubaker et al, 1973). It is possible that these two heptoses are characteristic of P.haemolytica as they are also present in A2 and A6 PWE (see section 4).

Reports by Sjewczyk and Taylor (1980) and Jann and Jann (1983) have indicated that the surface structures of S.typhi and E.coli respectively, are more complex than were first thought. It is now thought that individual components, such as KDO, previously thought to be mainly of LPS origin can belong to more than one type of molecule, either another LPS or a capsular polymer. If this type of diversity occurs widely throughout other bacterial genera then the difficulties facing any investigator are considerably increased. It may be that the explanation for the two distinct heptoses resides in the fact that there is a multiplicity of different polysaccharide molecules on the surface of P.haemolytica.

The question of purity is an important one and CIE analyses of A1 PWE shows that at least two antigens are present. The possibility that one of these is a loosely bound capsular polysaccharide is not substantiated by the finding that extraction with saline removes approximately the same proportions of the two antigens as does subsequent treatment with hot phenol-water.

Gentry et al (1982) reported that the capsule of P.haemolytica could be easily removed by phosphate buffered saline at 41°C.

However this capsular material contained protein and carbohydrate in a 5:4 ratio. If it is capsular material then it may be a glycoprotein similar to that described for Campylobacter fetus by Winter et al (1978).

Separation of the two antigens by immunological methods proved unsuccessful. Although the cross-reacting antigen could be clearly shown to precipitate with rabbit antiserum to A9 in an agarose gel it proved impossible to bind it successfully to antiserum or IgG bound to CNBr Sepharose 4B in an immunosorbent affinity column. This may have been due to the mixture of antigens in the solution or to leakage of the IgG from the column. Schurig et al (1978) found that in a study of Brucella abortus only two out of six identified antigens could be isolated by affinity chromatography and these two were protein antigens.

The attempt to isolate the antigens by selective precipitation of the cross-reacting antigen in agarose gel loaded with heterologous antiserum was more successful in that the cross-reacting antigen was stopped in the intermediate gel and purified serotype specific antigen could be collected. However, insufficient amounts of the antigen could be collected to allow successful GC analyses of the antigen. It would have been useful to have been able to identify the components of this antigen and use this information to postulate the possible origin of the antigen in the outer membrane. Winter et al (1978) described a microcapsule in Campylobacter fetus which is a glycoprotein complexed with LPS. This is not removed with saline but they postulate a requirement for cations, as EDTA

removes microcapsule from the LPS. A similar arrangement could be present on the surface of P.haemolytica A1.

### SECTION 3

The development and application of a serotype specific enzyme-linked immunosorbent assay (ELISA) for the detection of P.haemolytica antigens and antibodies.

Antibodies to P.haemolytica serotypes can be assayed by the IHA test (Biberstein et al, 1960) and also by the ELISA method described by Burrells et al (1978). The latter ELISA used SSE as the antigen and was shown to be more sensitive than the IHA test. However, the ELISA was not specific, in that it detected antibody to heterologous serotypes and Evans (1979) found a high degree of cross-reacting antibody in sera from rabbits and mice immunised with single serotype SSE vaccines. The demonstration that serotype-specific antigen can be purified by phenol-water extraction of SSE (Section 1) suggested that the use of this antigen in ELISA might improve the specificity of the test and increase its usefulness for the detection of antibody to specific serotypes.

SECTION 3.1

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The demonstration of the type-specific antigenicity of *P.haemolytica* A1 PWE in the indirect enzyme linked immunosorbent assay.

Inhibition of specific immune reactions has been shown to be an effective indicator of the inhibitor's participation as an antigen in the reaction. This has been described for *E.coli* "O" antigen-typing schemes (Orskov et al, 1977) where absorption of sera with different antigenic types allows the production of monospecific sera which can then be used to identify that antigenic determinant on strains of unknown antigenic status.

As A1 PWE has been shown to contain the serotype antigen by IHA and CIE (Section 1) it might therefore be worth testing it as a more specific antigen in an ELISA system.

The ELISA method described by Burrells et al (1979), which used SSE as the antigen, combined with various absorption procedures on sheep anti A1 serum, should indicate whether the serotype specificity of A1 PWE is acceptable as an antigen in the ELISA system.

SSE's of *P.haemolytica* A1, A2, A6, A7, A9, T10 and UT6 and the PWE of SSE of A1, A2, A6 and A9, were prepared as described in Materials and Methods.

Antisera were raised in specific pathogen free (SPF) lambs by immunising with A1 SSE antigen as described in Materials and Methods

Rabbit antisera were prepared as described in Materials and Methods.



Absorption of antisera - Sheep antiserum against Al SSE, obtained from SPF lambs, was absorbed with heterologous SSE's (2.5mg/ml) or Al PWE (5mg/ml) or both as described in Table 3.1. The suspensions were mixed for 2h at 37°C then left overnight at 4°C. The precipitates were removed by centrifugation at 11000g for 5 min.

The ELISA method is described in Materials and Methods.

The A405 readings obtained with the four antiserum preparations when titrated against Al and All SSE antigens are shown in Figures 3.1 and 3.2 respectively. The effect of absorption with heterologous SSE antigens was to reduce the A405 only slightly when reacted with Al SSE but to reduce substantially the reaction against All SSE (titre of 1 in 30,000 and 1 in 3000 respectively). Absorption with Al PWE almost halved the initial A405 of unabsorbed serum (0.57 and 1.0 respectively) when reacted against Al SSE but little effect was seen with All SSE. The combination of SSE's and Al PWE almost removes completely the reactivity of the serum for Al SSE. The same combination cannot be differentiated from serum absorbed with heterologous SSE's when the reaction against All SSE was examined.

The results confirmed the earlier findings with IHA and CIE that Al PWE contains the type-specific antigen and that it is in a fairly pure antigenic form as it does not decrease the response of anti Al serum to the heterologous All SSE antigen but does decrease the response to homologous antigen. It is therefore a suitable antigen to test for serotype specificity in the ELISA system.

Table 3.1

Mixtures used for absorption of antiserum to P.haemolytica A1 with heterologous SSE and homologous PWE.

SAMPLE	SERUM VOLUME	SSE	A1	PWE	PBS	TOTAL	DILUTION OF SERUM
1	0.1ml	0.2ml each of A2, A6,A7,A9,T10,UT6	-		4.7ml	6.0ml	1 in 60
2	0.1ml	0.2ml each of A2, A6,A7,A9,T10,UT6	0.2ml		4.5ml	6.0ml	1 in 60
3	0.1ml	-	0.2ml		5.7ml	6.0ml	1 in 60
4	0.1ml	-	-		5.9ml	6.0ml	1 in 60

Figure 3.1

Absorbance at 405nm (A405) of dilutions of SPF lamb antisera, raised against P.haemolytica A1 reacted with homologous A1 SSE antigen in ELISA. The antiserum was absorbed with heterologous SSE's (O), heterologous SSE's and A1 PWE (●), A1 PWE (□) or not absorbed (■).

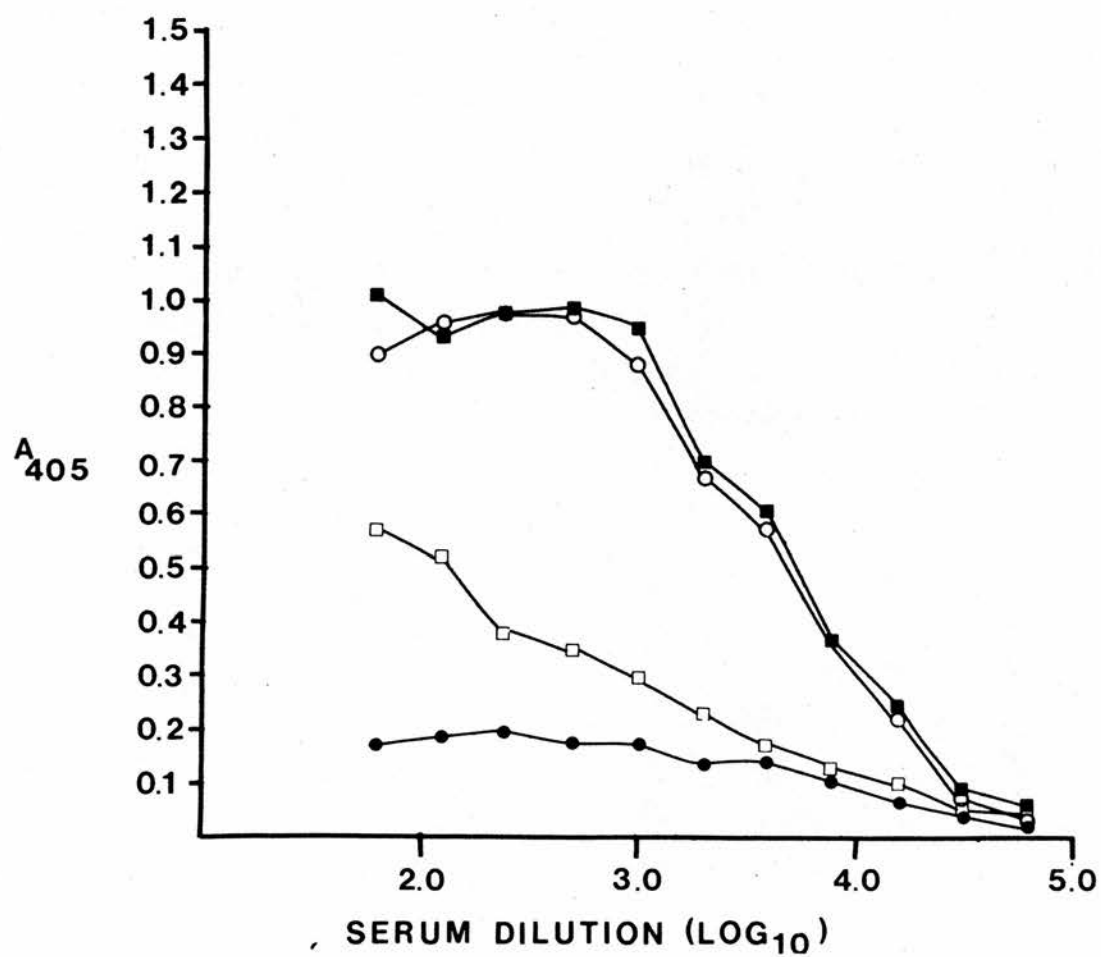
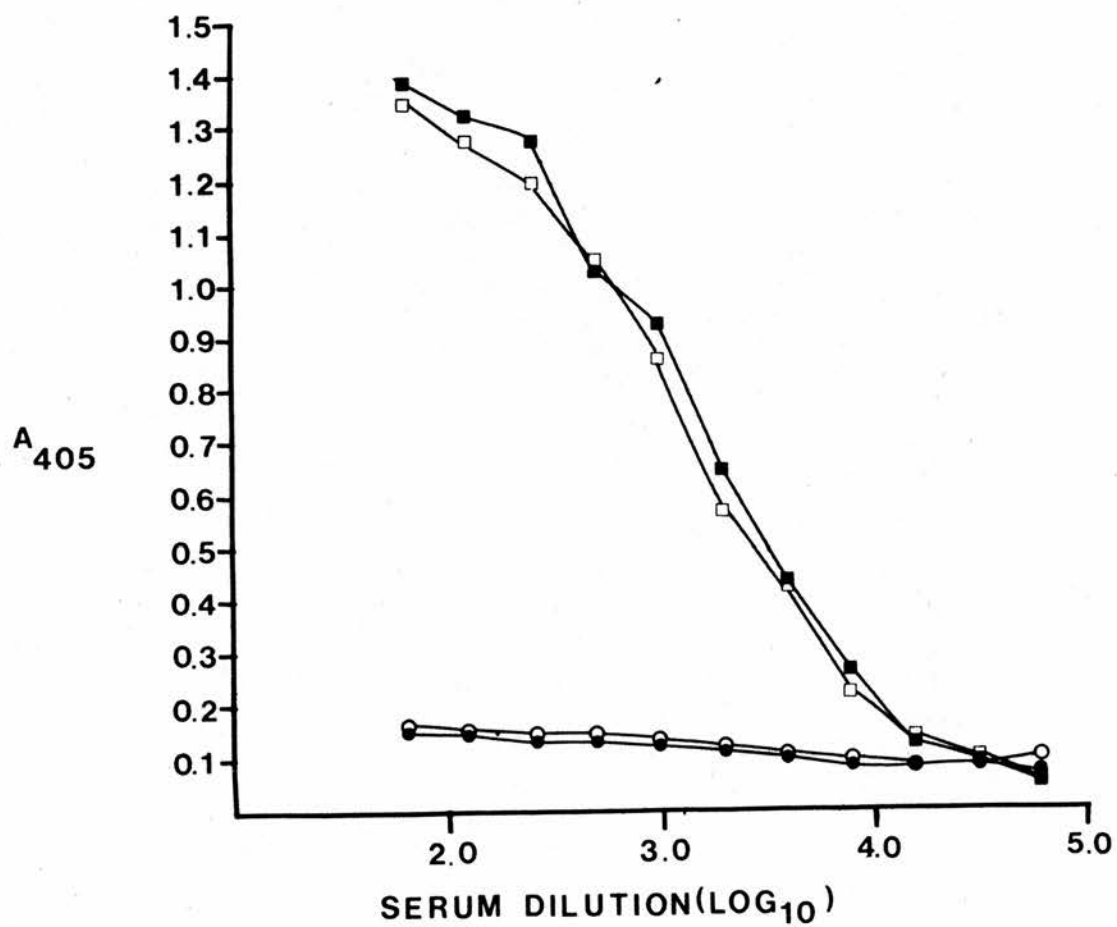


Figure 3.2

Absorbance at 405nm (A405) of dilutions of SPF lamb antisera raised against P.haemolytica A1 reacted with heterologous All SSE in ELISA. The antiserum was absorbed with heterologous SSE's (O), heterologous SSE's and A1 PWE (●), A1 PWE (□) or not absorbed (■).



SECTION 3.2Development of a serotype specific ELISA system for  
*P.haemolytica*

The previous experiment demonstrated that Al PWE was a potential candidate for a specific serotype antigen for use in the ELISA system. The effectiveness of an ELISA depends on the use of optimal concentrations of all reagents which have to be determined before the test can be employed routinely. This experiment describes the standardisation of the various reagents.

The preparation of antigens, sheep and rabbit antisera, rabbit IgG and the ELISA method have been described in Materials and Methods.

Titration of Al PWE as an antigen: Al PWE ( $1\text{mg ml}^{-1}$ ) was diluted from 1 in 10 to 1 in 10,000 in ten fold steps and allowed to bind to wells for 18h at  $4^{\circ}\text{C}$ . The result, when the ELISA was performed as in Materials and Methods, was negative. No colour developed in any wells except for a control well where conjugate and substrate were mixed indicating a reaction between these two reagents. The sheep serum had previously been used successfully in gel immunoelectrophoresis and therefore recognised Al PWE. It also contained IgG antibody to Al PWE as it had reacted with the conjugate in the previous experiment (3.1). A possible explanation for the result was the failure of the antigen to bind to the plate.

### 3.2.1. The use of whole rabbit antiserum as a trapping layer in a "sandwich" ELISA

It has been reported that difficulties are sometimes encountered when attempts are made to bind some bacterial polysaccharide antigens to plastic for use in the ELISA system (Russell et al, 1980). This can be overcome by using a sandwich ELISA, where antiserum is bound to the plastic in an initial step and this traps the antigen for the test. This has been applied successfully by Barrett et al (1980) for the trapping of pneumococcal antigens.

Rabbit antiserum against P.haemolytica A1 whole cells was diluted 1 in 50 in antigen buffer and added to all wells of an ELISA plate. This was left for 18h at 4°C. After washing, A1 PWE ( $1\text{mg ml}^{-1}$ ) diluted from 1 in 50 to 1 in 800 in PBS/Tween 20 was added to the plate and the plate left at 20°C for 3h. Subsequent steps were identical to those described in Materials and Methods except that sheep antiserum to P.haemolytica A2 was included in the antiserum step as a heterologous antiserum control.

The result is shown in Table 3.2. The A405 for the homologous reaction was very low (0.429-0.525) compared to the expected result of 1.0 or greater. It is possible that, when using whole serum, other serum components such as albumin bind to the plastic wells more strongly than immunoglobulins. If this is the explanation then a purified IgG fraction would be expected to bind to the wells more effectively and thereby increase the amount of antigen bound in the second step. The absorbances obtained with the heterologous antiserum (A2)



indicated that either the antigen was not totally specific or that non-specific binding was occurring.

Table 3.2

Absorbance readings at 405nm (A405) for ELISA reaction between P.haemolytica A1 PWE with sheep anti A1 and A2 conjugates and a rabbit antiserum against P.haemolytica A1 whole cells to trap antigen.

SHEEP ANTISERUM	A405 READINGS AT THE FOLLOWING DILUTIONS OF A1 PWE				
	50	100	200	400	800
Anti-A1	0.429	0.540	0.525	0.547	0.479
Anti-A2	0.214	0.234	0.217	0.178	0.170

### 3.2.2. The Use of the IgG fraction from a rabbit antiserum to *P.haemolytica* A1 as a trapping layer in a "sandwich" micro ELISA

The initial step in ELISA, that of binding antigen to plastic is little understood but it seems obvious that the purer the preparation then the more likelihood there is of adequate amounts of the antigen binding to the plastic. The use of a purified IgG fraction of a rabbit antiserum to *P.haemolytica* A1 should allow the binding of a much greater amount of IgG than would occur in the presence of other serum constituents.

The IgG fraction from a rabbit antiserum to *P.haemolytica* A1 whole cells ( $4.9\text{mg ml}^{-1}$ ) diluted in 1 in 100 in antigen buffer, was added to all wells (i.e.  $16\mu\text{g}$  IgG per well) and left at  $4^{\circ}\text{C}$  for 18h.

Antigens A1, A2 and A6 PWE (all at  $1\text{mg ml}^{-1}$ ) were diluted in doubling dilutions from 1 in 100 to 1 in 51,200 in PBS/Tween, added to the plate and left at  $4^{\circ}\text{C}$  for 18h.

The remaining steps were identical to those given for the ELISA in part 1 of this experiment.

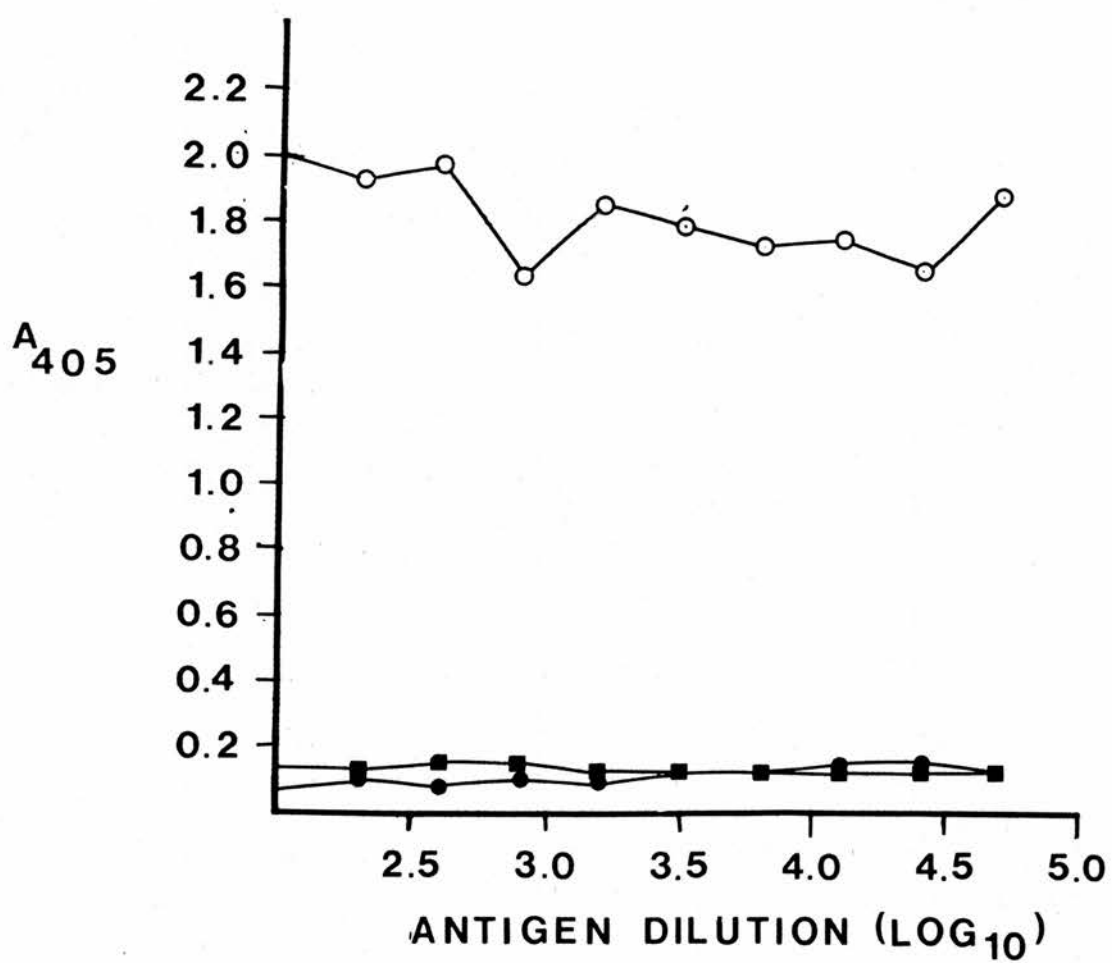
The A405 reading obtained for the dilutions of A1 PWE reacted with homologous and heterologous antisera is displayed graphically in Figure 3.3. Control wells exposed to the rabbit IgG, antiserum conjugate and substrate but no antigen, did not show colour development.

The results show that the homologous reaction is very strong (A405 between 2 and 1.63) with no loss of antigen activity even at the 1 in 51,200 dilution of antigen. The specificity of the

reaction is confirmed by the very low A405 obtained with the heterologous antigens (no A405 above 0.15 for either A2 or A6).

**Figure 3.3**

Absorbance at 405nm (A405) of various dilutions of PWE antigens from P.haemolytica A1(O), A2(●), or A6(■) reacted with the IgG fraction of a rabbit antiserum and SPF lamb antisera, raised against A1, in a "sandwich" ELISA.



### 3.2.3 The checkerboard titration of IgG fractions of rabbit antisera to A1, A2, A6 and A9 and prospective PWE antigens in a "sandwich" ELISA

The demonstration of effective binding of A1 PWE by the IgG fraction from a rabbit antiserum to A1 suggested that the same system might be applied to other serotypes of P.haemolytica. The optimum concentration of IgG and PWE for use in the test was assessed by checkerboard titration. This was carried out as follows; 1)The IgG fractions of rabbit antisera to A1, A6 and A9 were diluted in antigen buffer from 1 in 500 to 1 in 8000 and IgG fractions against A2 diluted from 1 in 100 to 1 in 1600. After the addition of the dilutions to the wells the plates were left at 4°C for 18h. 2)Ten fold dilutions of A1, A2, A6 and A9 PWE antigens ( $1\text{mg ml}^{-1}$ ) from 1 in 100 to 1 in 100,000 in PBS/Tween were added to the wells and left at 4°C for 18h. 3)Antisera raised in SPF lambs against each serotype were diluted 1 in 500 with diluent and added to the appropriate wells. The plates were left at RT for 3h. The remaining steps were the same as described previously.

The titrations of rabbit IgG and PWE for A1, A6 and A9 are shown in Tables 3.3, 3.4 and 3.5 respectively. The A2 reaction did not give any colour development whereas the A1, A6 and A9 components did react and Table 3.6 shows the combination of dilutions of IgG and PWE which give an A405 of around 1.0 for each serotype. This A value was arbitrarily selected as a convenient standard. With these dilutions it should be possible to measure antibody to these three serotypes in the sera of sheep. The failure of the A2 reaction may have been due to

insufficient or low affinity IgG in the coating layer or to insufficient binding of antigen or it may have been due to some degradation of the A2 antigen by phenol-water treatment.



Table 3.3

Checkerboard titration of anti-A1 rabbit IgG and A1 PWE.

DILUTIONS OF A1 PWE	A405 READINGS AT THE FOLLOWING DILUTIONS OF RABBIT IgG				
	500	1000	2000	4000	8000
100	2.25	2.0	2.0	1.25	0.75
1000	1.0	1.0	1.0	0.75	0.25
10000	0.25	0.25	0.25	0.00	0.00
100000	0.00	0.00	0.00	0.00	0.00

Table 3.4

Checkerboard titration of anti-A6 rabbit IgG and A6 PWE

DILUTIONS OF A1 PWE	A405 READINGS AT THE FOLLOWING DILUTIONS OF RABBIT IgG				
	500	1000	2000	4000	8000
100	1.4	1.4	1.2	0.8	0.00
1000	0.4	0.6	0.6	0.2	0.00
10000	0.4	0.4	0.4	0.0	0.00
100000	0.4	0.4	0.4	0.0	0.00

Table 3.5

Checkerboard titration of anti-A9 rabbit IgG and A9 PWE.

DILUTION OF A9 PWE	A405 READINGS AT THE FOLLOWING DILUTIONS OF RABBIT IgG				
	500	1000	2000	4000	8000
100	1.8	1.6	1.4	1.2	0.6
1000	1.6	1.4	1.4	1.0	0.4
10000	1.2	1.0	1.0	0.8	0.4
100000	0.6	0.6	0.4	0.4	0.2

Table 3.6

Dilutions of IgG and PWE to be used in ELISA for P.haemolytica serotypes A1, A6 and A9.

SEROTYPE	DILUTION	
	IgG (5mg ml <sup>-1</sup> )	PWE (1mg ml <sup>-1</sup> )
A1	1000	1000
A6	1000	100
A9	1000	10000

### SECTION 3.3

#### The use of the micro ELISA to detect antibodies to specific *P.haemolytica* serotypes in the sera of SPF lambs.

The micro ELISA for measurement of antibodies in sheep sera to *P.haemolytica*, described by Burrells *et al* (1979), employed SSE as the antigen but Evans (1979) showed that there was much cross-reactivity between serotypes when this antigen was used in ELISA to measure antibody in mouse or rabbit sera to different serotypes. The PWE used in the "sandwich" ELISA should substantially decrease the cross-reactivity experienced in earlier methods. The use of SPF lambs to raise serotype specific antisera should overcome the problem of prior exposure by experimental animals to a range of *P.haemolytica* serotypes.

Sera from two unvaccinated control lambs and from two groups of 3 lambs vaccinated with either A1 or A9 SSE taken before vaccination and 6 weeks after vaccination were diluted 1 in 1000 for use in the ELISA. The micro ELISA used was that described in experiment 3.2 with the A1 and A9 reagents at optimum dilutions. The sera were reacted against homologous and heterologous antigens.

Table 3.7 shows the result obtained when antisera raised to A1 and A9 in SPF lambs were tested against homologous and heterologous PWE antigens. Individual homologous values were all greater than 0.69 whilst heterologous values were less than 0.23. Paired sera from the two unvaccinated lambs remained negative to A1 PWE, but showed some reaction to A9 PWE. This reaction was also seen in the pre-vaccination sera of vaccinated lambs.

The results corroborate these found in experiment 3.2 in which the sandwich ELISA, incorporating A1 PWE as antigen, measured antibody to specific serotype antigens in the sera of sheep. The criterion of specificity is that used by Russel et al (1980) to measure antibody to pneumococcal antigen; i.e. the lowest A405 in a homologous system (lamb3 serum = 0.691) was more than three times greater than the highest heterologous A405 (lamb 2 serum = 0.225). The level of reactivity with A9 PWE of both prevaccinated and control sera suggests that the lambs may have been exposed to some cross-reacting antigen derived from an organism other than P.haemolytica. Similar cross-reactions with P.haemolytica A1 antigens have been seen in IHA tests on the sera of SPF lambs which had not experienced P.haemolytica infection as far as could be determined ( Dr. N.J.L. Gilmour, personal communication).

Table 3.7

Values obtained from the reaction of antisera to type A1 or A9 with homologous and heterologous phenol-water extracted antigens.

SPF Lamb No.	VACCINES	A405 VALUES			
		A1 PWE		A9 PWE	
		Pre-vac	Post-vac	Pre-vac	Post-vac
1	A1 SSE	0	1.065	0.097	0.122
2		0	1.087	0.132	1.225
3		0	0.691	0.126	0.154
4	A9 SSE	0	0.025	0.096	1.140
5		0	0	0.117	0.865
6		0	0.030	0.084	0.696
7	Not Vaccinated	0	0	0.086	0.151
8		0	0	0.117	0.130

SECTION 3.4The development of a micro ELISA to measure antibody to  
P.haemolytica antigens in mouse serum.

The mouse model for P.haemolytica infection, described by Evans and Wells (1979a) offers a method for the initial testing of potential pasteurella vaccine components where the efficacy of the antigen in providing protection against P.haemolytica challenge can be assessed. However, the model is subject to biological variation and the responses to challenge in any one group of vaccinated animals can differ greatly. The detection of antibodies against P.haemolytica in individual mouse serum would allow assessment of the reaction of the mouse to the antigens and possibly answer the question whether antibodies are necessary for protection against challenge. This experiment describes the development of an ELISA to measure antibody to P.haemolytica in the sera of immunised and non-immunised mice.

### 3.4.1 Titration of goat anti-mouse IgG, conjugated with alkaline phosphatase, and sera from vaccinated mice.

Antisera were raised in three groups of C57 black mice by subcutaneous inoculation of 0.1 ml of Al SSE (125 ug per dose) or 0.1 ml of a PWE (50ug/dose) in CFA. These inoculations were repeated 14 days after the initial dose. Fourteen days after the second dose the mice were exsanguinated by cardiac puncture and the sera from the five mice in each group were pooled. A control group of 5 unvaccinated mice were killed and exsanguinated at the same time and their sera pooled.

ELISA. The IgG fraction of a rabbit antiserum against Al whole cells and Al PWE were added to wells in a micro ELISA plate as previously described in experiment 3.2. Mouse anti-sera and goat anti-mouse IgG conjugated with alkaline phosphatase (Miles Laboratory, Slough, U.K.) were checkerboard titrated; mouse serum was diluted in ten fold dilutions from 1 in 10 to 1 in 100000, added to micro ELISA plates and left for 3h at RT. Goat anti-mouse IgG conjugate diluted 1 in 50, 100, 400 and 800 was added to the wells and left for 3h at RT. Substrate was reacted for 1h before 3M NaOH was added to stop the reaction. Absorbances were read as previously described.

The A405 measured for the reaction of mouse anti-Al PWE sera and mouse anti-Al SSE sera are shown in Tables 3.8 and 3.9 respectively. No A405 readings were obtained for the control pool of serum indicating that there was very low background reaction in the test.

The results suggested a working dilution of 1 in 500 for serum and 1 in 200 for conjugate which in both groups would give

an A405 approaching the previously selected standard of 1.0. The results also showed that A1 PWE does stimulate antibody response in vaccinated mice.



Table 3.8

Titration of mouse antiserum against P.haemolytica Al PWE and goat antiserum against mouse IgG conjugated with alkaline phosphatase in an indirect "sandwich" micro ELISA against Al PWE antigen.

DILUTION OF CONJUGATE	ABSORBANCE (A405) READINGS AT THE FOLLOWING DILUTION OF SERUM				
	10	100	1000	10000	100000
50	2.0	2.0	1.07	0.41	0.45
100	2.0	2.0	0.46	0.26	0.5
400	0.77	0.97	0.86	0.04	0.07
800	0.39	0.48	0.00	0.00	0.1

Table 3.9

Titration of mouse antiserum against P.haemolytica Al SSE and goat antiserum against mouse IgG conjugated with alkaline phosphatase in an indirect" sandwich" micro ELISA against Al PWE antigen.

DILUTION OF CONJUGATE	ABSORBANCE (A405) READINGS AT THE FOLLOWING DILUTION OF SERUM				
	10	100	1000	10000	100000
50	2.0	2.0	2.0	0.75	0.90
100	1.29	1.9	1.27	0.15	0.10
400	0.34	0.7	0.39	0.02	0.03
800	0.12	0.35	0.15	0.00	0.00

### 3.4.2. Sequential measurement of antibodies to P.haemolytica

#### Al antigens in the blood of mice vaccinated with Al PWE or SSE

The above titration in section 3.4.1 indicated that antibodies to P.haemolytica Al could be measured in mouse serum samples. However, for the sequential measurement of antibody in individual mice it is difficult to remove sufficient volumes of blood to allow the collection of enough serum.

The following experiment was designed to investigate whether whole blood could be used directly in the ELISA.

Three groups of 5 mice treated in the same way as described in the first part of this experiment (3.4.1) were killed and 50ul of blood, taken from the axilla of each mouse, were diluted 100 fold in PBS Tween. This assumed that a 1 in 100 dilution of whole blood was equivalent to a 1 in 200 dilution of serum. In the ELISA the IgG fraction of rabbit anti serum raised against Al whole cells IgG, Al PWE and Al SSE antigens were added as previously described. Mouse blood samples were diluted further to 1 in 200 and 1 in 500 in PBS/Tween, added to the plates in duplicate wells and left at RT for 3h. Conjugate was added at a 1 in 200 dilution and the plate incubated at RT for 3h. Substrate was added to every well and the reaction stopped after 1h by the addition of 3M NaOH. The A405 readings were measured as previously described.

The A405 readings obtained for two dilutions of blood taken from vaccinated or unvaccinated mice reacted with Al PWE or SSE are shown in Table 3.10.

The results show that antibodies to P.haemolytica can be

measured in whole blood samples of mouse blood and that little background activity is present although the A405 readings obtained for mouse 15 (0.41 and 0.31) were high. It would also appear that the response to A1 PWE vaccination was not as strong as the response to A1SSE given only 2 out of 5 mice vaccinated with PWE responded with A405 readings above 0.5 (numbers 7 and 9) whereas all mice vaccinated with SSE did respond to PWE with A405 above 0.5.

The use of whole blood sampling should facilitate the monitoring of the mouse immune response to vaccination with P.haemolytica antigens.

Table 3.10

A405 readings obtained with dilutions of whole blood taken from mice vaccinated with P.haemolytica A1 SSE or PWE reacted against A1 SSE or PWE antigens in an indirect "sandwich" ELISA .

MOUSE	VACCINE	DILUTION OF BLOOD	A405 READINGS WITH THE FOLLOWING ANTIGENS	
			SSE	PWE
1	A1 SSE	200	1.92	1.87
	A1 SSE	500	1.33	1.19
2	A1 SSE	200	1.23	1.17
	A1 SSE	500	0.55	0.92
3	A1 SSE	200	1.70	1.1
	A1 SSE	500	0.74	0.68
4	A1 SSE	200	1.97	1.23
	A1 SSE	500	1.46	1.00
5	A1 SSE	200	1.35	1.14
	A1 SSE	500	0.50	0.66
6	A1 PWE	200	1.11	0.30
	A1 PWE	500	0.40	0.08
7	A1 PWE	200	1.09	0.60
	A1 PWE	500	0.32	0.23
8	A1 PWE	200	0.35	0.23
	A1 PWE	500	0.18	0.08
9	A1 PWE	200	0.96	1.02
	A1 PWE	500	0.36	0.49
10	A1 PWE	200	1.18	0.39
	A1 PWE	500	0.58	0.16
11	UNVACCINATED	200	0	0.08
	UNVACCINATED	500	0	0.07
12	UNVACCINATED	200	0	0
	UNVACCINATED	500	0	0
13	UNVACCINATED	200	0.21	0.06
	UNVACCINATED	500	0	0.04
14	UNVACCINATED	200	0	0.07
	UNVACCINATED	500	0	0.01
15	UNVACCINATED	200	0.02	0.41
	UNVACCINATED	500	0	0.32

SECTION 3.5

The use of ELISA to measure sequentially antibody to *P.haemolytica* in the blood of mice vaccinated with Al PWE and SSE antigens and the correlation of those measurements with protection against challenge with *P.haemolytica* Al.

PWE antigen of *P.haemolytica* Al has already been shown to protect mice against homologous challenge (Section 1.2). However, in those experiments not all mice in the vaccinated groups were protected to the same degree against challenge suggesting that individual mice had responded differently to vaccination.

Measurement of antibody titres to *P.haemolytica* present after vaccination and correlation of these with protection against challenge should give some indication whether the above explanation is correct.

Al SSE and Al PWE were emulsified in CFA to give a final concentration of 62.5ug and 6ug per dose of vaccine respectively.

Two groups of ten randomly allocated C57 black mice were inoculated subcutaneously with 0.1ml of SSE or PWE vaccine and revaccinated 14 days later. Another group of ten mice were left as unvaccinated controls. Fourteen days after the second vaccination the mice were challenged with *P.haemolytica* Al as described in Materials and Methods. The group results were analysed statistically by the Mann Whitney Ranking Test. Samples of mouse blood were obtained by pricking the tail vein and withdrawing 10ul of blood which were immediately diluted in 1ml

of PBS/Tween. The mice were sampled before vaccination, at revaccination and one day before challenge and the diluted blood samples were stored at  $-20^{\circ}\text{C}$ . Each mouse was individually identified.

Antibodies to P.haemolytica A1 SSE and PWE were detected by the following ELISA method. The plates were washed between steps with PBS/Tween.

1)The IgG fraction of a rabbit anti serum raised against P.haemolytica A1 whole cells was diluted 1 in 500 in antigen buffer, added to the wells and left at  $4^{\circ}\text{C}$  for 18h.

2)A1 PWE at 1 in 1000 dilution and A1 SSE at 1 in 500 dilution in PBS/Tween were added to appropriate wells and left at  $4^{\circ}\text{C}$  for 18h.

3)Standard mouse sera pooled from a previous experiment (Exp 3.4.1 part 1) was doubly diluted in PBS/Tween from 1 in 100 to 1 in 12800 and added to columns 2 and 3 in the micro ELISA plate. Test blood samples were diluted 1 in 2 giving a 1 in 200 dilution of the blood and added to duplicate wells for 3h at RT.

4)Goat anti-mouse IgG conjugated with alkaline phosphatase was diluted 1 in 200 in PBS/Tween, added to all wells and left at RT for 3h.

5)Substrate, N-para nitro phenyl phosphate ( $1\text{mg ml}^{-1}$ ) was added to all wells for 1h at RT. The reaction was stopped by the addition of 3M NaOH and the A405 readings taken as described previously.

Calculation of results:

1)The A405 readings (A) were converted to  $\log_{10}$  values.

2)The log A values of the standard serum dilutions were plotted against the  $\log_{10}$  values of these dilutions to provide a standard graph.

3)The titre of the standard serum was taken to be that dilution which would give an A405 value of just greater than 0.1, as negative control sera taken from SPF and gnotobiotic lambs never exceeded this value.

4)The titres of the test sera are calculated from the formula,

$$\text{Titre of test serum} = \frac{\text{Dilution of test serum (200)} \times \text{Titre of standard serum}}{\text{Dilution of standard serum which would give the same A value as the test serum}}$$

The correlation between the counts of P.haemolytica in the liver and the serum antibody titres against A1 SSE and PWE antigens for individual mice are given in Tables 3.11 and 3.12 respectively. All mice vaccinated with SSE or PWE showed rising titres from the time of first vaccination to challenge against both antigens. Taken as groups, all vaccinated mice were protected against challenge to a significant level,  $P > 0.001$  for both SSE and PWE vaccinated groups compared to the unvaccinated control group. Unvaccinated control mice had no detectable antibody to P.haemolytica although two mice in the group (24 and



25) had low liver counts. The titres obtained against SSE were generally higher than those against PWE no matter what the vaccine. This probably reflects the greater variety of antigens present in SSE although direct comparisons between values obtained in the two ELISA's are difficult to interpret. The average titres for each vaccinated group against PWE before challenge and the protection obtained are not significantly different.

This demonstrates that vaccination with a purified antigen, PWE, at one tenth of the concentration of the SSE gives the same antibody titre and protection as obtained with the SSE.

Table 3.11

Antibody titres against A1 SSE determined by ELISA and viable count of bacteria in the livers of mice challenged with P.haemolytica A1.

MOUSE No	VACCINE STATUS	ANTIBODY			TITRE	CHALLENGE SEROTYPE	LIVER COUNT (cfu) <sup>1</sup>
		DAY1	DAY14	DAY28			
1	A1 SSE	-	215	1406	A1 <sup>2</sup>	3.8	
2	A1 SSE	-	-	3355	A1	2.7	
3	A1 SSE	-	204	1940	A1	2.7	
4	A1 SSE	-	-	949	A1	2.7	
5	A1 SSE	-	-	7709	A1	2.7	
6	A1 SSE	-	413	15757	A1	3.3	
7	A1 SSE	-	464	1805	A1	3.0	
8	A1 SSE	-	-	1303	A1	2.94	
9	A1 SSE	-	-	756	A1	3.57	
10	A1 SSE	-	277	550	A1	2.7	
11	A1 PWE	-	270	1295	A1	2.7	
12	A1 PWE	-	267	1022	A1	2.7	
13	A1 PWE	-	345	1756	A1	3.0	
14	A1 PWE	-	-	432	A1	2.7	
15	A1 PWE	-	228	681	A1	2.7	
16	A1 PWE	-	-	1162	A1	3.0	
17	A1 PWE	-	220	3539	A1	2.7	
18	A1 PWE	-	217	2928	A1	2.7	
19	A1 PWE	-	-	325	A1	2.7	
20	A1 PWE	-	212	649	A1	2.7	
21	CONTROL	-	-	-	A1	6.11	
22	CONTROL	-	-	-	A1	7.18	
23	CONTROL	-	-	-	A1	6.18	
24	CONTROL	-	-	-	A1	3.0	
25	CONTROL	-	-	-	A1	3.76	
26	CONTROL	-	-	-	A1	5.68	
27	CONTROL	-	-	-	A1	6.68	
28	CONTROL	-	-	-	A1	6.57	
29	CONTROL	-	-	-	A1	7.08	
30	CONTROL	-	-	-	A1	6.08	

(1)-COLONY FORMING UNITS ( $\log_{10}$  values)

(2)-CHALLENGE DOSE =  $5.2 \times 10^6$  cfu

Table 3.12

Antibody titres against A1 PWE determined by ELISA and viable counts of bacteria in the livers of mice challenged with P.haemolytica A1.

MOUSE No.	VACCINE	ANTIBODY TITRE			CHALLENGE SEROTYPE	LIVER COUNT cfu <sup>1</sup>
		DAY1	DAY14	DAY28		
1	A1 SSE	-	201	1034	A1	3.8
2	A1 SSE	-	-	903	A1	2.7
3	A1 SSE	-	-	1226	A1	2.7
4	A1 SSE	-	-	599	A1	2.7
5	A1 SSE	-	-	756	A1	2.7
6	A1 SSE	-	389	1811	A1	3.3
7	A1 SSE	-	401	1004	A1	3.0
8	A1 SSE	-	-	400	A1	2.94
9	A1 SSE	-	-	662	A1	3.57
10	A1 SSE	-	232	319	A1	2.7
11	A1 PWE	-	252	2358	A1	2.7
12	A1 PWE	-	269	329	A1	2.7
13	A1 PWE	-	299	425	A1	3.0
14	A1 PWE	-	-	266	A1	2.7
15	A1 PWE	-	-	455	A1	2.7
16	A1 PWE	-	-	224	A1	3.0
17	A1 PWE	-	-	1030	A1	2.7
18	A1 PWE	-	-	7274	A1	2.7
19	A1 PWE	-	-	219	A1	2.7
20	A1 PWE	-	-	253	A1	2.7
21	CONTROL	-	-	-	A1	6.11
22	CONTROL	-	-	-	A1	7.18
23	CONTROL	-	-	-	A1	6.18
24	CONTROL	-	-	-	A1	3.00
25	CONTROL	-	-	-	A1	3.76
26	CONTROL	-	-	-	A1	5.68
27	CONTROL	-	-	-	A1	6.68
28	CONTROL	-	-	-	A1	6.57
29	CONTROL	-	-	-	A1	7.08
30	CONTROL	-	-	-	A1	6.08

1. Log<sub>10</sub> values

2. Challenge dose = Log<sub>10</sub> 6

- = negative value

### SECTION 3.6

The development of an ELISA to measure antibody in sheep serum to P.haemolytica A2 antigens.

P.haemolytica A2 is the serotype most frequently isolated from ovine enzootic pneumonia (Fraser et al, 1982a) and therefore control of the disease depends on immunity to this serotype. However, the A2 serotype is one of the least immunogenic of the P.haemolytica serotypes and in rabbits the production of antiserum for serotyping A2 strains has to be carried out with live inoculation of bacterial cells (Fraser et al, 1982a). In sheep there is no measurable antibody response in the IHA test after vaccination with killed cells or SSE but there is a marked response after challenge with live organisms (Dr. N.J.L. Gilmour, personal communication).

The results of earlier experiments (Section 3.2) have shown that the IgG binding layer (sandwich) technique was not effective in binding A2 PWE to the wells of a micro ELISA plate. This may have been due to the difficulty in producing high titres of antibody against A2 in rabbits which would reduce the effectiveness of any IgG fraction.

Dahlberg and Branefors (1980) have overcome the problem of binding an LPS antigen from Haemophilus influenzae directly to plastic by extending the time the antigen is in contact with the plastic. Their method was applied to the A2 PWE antigen.

The standard antiserum used was a pool of conventional sheep sera from animals shown to have a high titre in the IHA test against A2 antigen.

A2 PWE ( $1\text{mg ml}^{-1}$ ) in doubling dilution from 1 in 50 to 1 in 6400 in antigen buffer was added to duplicate wells in a micro ELISA plate and incubated at  $37^{\circ}\text{C}$  for 24h followed by storage at  $4^{\circ}\text{C}$  for 7 days. After washing, the standard serum, diluted 1 in 500, was added to the plate and left at RT for 3h. The remaining steps were as described in Materials and Methods.

The dilution of antigen giving an A405 reading of 1.0 was a 1 in 200 dilution. By this method it should be possible to measure the antibody levels to A2 PWE in the sera of sheep. The dilution of PWE used is substantially lower than that used on the A1 ELISA (1 in 1,000 dilution) but greater than that calculated for A6 (1 in 100).

### SECTION 3.7

The measurement of antibody levels to *P.haemolytica* A2, by IHA and ELISA, in the sera of sheep with experimental chronic pasteurellosis.

Atypical or chronic pneumonia, caused by *P.haemolytica* in conjunction with *Mycoplasma ovipneumonia* has been reproduced experimentally (Jones et al, 1978) and because the sheep undergo an active infection the antibody response should be measurable during the course of this infection. The system also offers the opportunity to compare the ELISA for A2, described in the previous experiment, with the IHA test.

*P.haemolytica* A2 was grown in nutrient broth for 18h at 37°C. Twenty eight lambs, approximately 10 weeks old, were obtained from a flock in which levels of infection with potentially pathogenic respiratory organisms had been found to be very low over 4 years of sampling. Nasal swabs taken from the lambs on day 1 were free of *P.haemolytica* and mycoplasmas.

Eighteen lambs were inoculated intratracheally on day 1 with 6ml of a homogenate of pneumonic lung lesion obtained from the abattoir (Jones et al, 1978). This homogenate contained  $10^6$  colour changing units (ccu) per 0.2ml of *M.ovinpneumoniae* and  $2 \times 10^3$  cfu per 0.2ml of *P.haemolytica* A2 with no isolation of any other micro-organism. The same animals were inoculated intranasally on day 7 with 1ml of a culture of *P.haemolytica* A2 containing  $4 \times 10^6$  cfu ml<sup>-1</sup>. The remaining 10 control animals were untreated throughout. All control lambs and 6 infected lambs were slaughtered at 15.5 weeks post infection (wpi), and

the surviving 8 infected lambs were slaughtered at 24.5 wpi, when they had reached the same mean liveweight as the controls. These procedures were carried out by Dr. G.E. Jones, Moredun Research Institute.

All lambs were bled before challenge and at fortnightly intervals thereafter.

Sera from sheep shown to have antibody to P.haemolytica A2 by the IHA test in a previous experiment were pooled and stored in aliquots at -20°C until used as a standard positive antiserum. Antisera to P.haemolytica A1 and A9 were prepared in SPF lambs as described in Materials and Methods.

The IHA test is described in Materials and Methods.

The ELISA was carried out as follows;

1) Micro ELISA plates were coated with A2 PWE diluted 1 in 200 in antigen buffer and inoculated at 37°C for 24h then held at 4°C for 7 days.

2) Standard or test sera were added to all wells except those in column 1 to which diluent only was added. Test sera were diluted 1 in 2000 in PBS/Tween + 0.02% sodium azide before use. The standard A2 antiserum was made up in doubling dilutions from 1 in 2,000 to 1 in 256,000. The A1 and A9 antisera were diluted in 1 in 2,000. Each sample was tested in duplicate and the plates left at RT for 3h.

The remaining steps were those described in Materials and Methods. Titres were calculated by the formula given in Section 3.5.

The ELISA results of the calculated mean serum titres against P.haemolytica for infected and control animals are shown in

Figure 3.4. Mean antibody titres against P.haemolytica in the infected group reached their maximum at 8pwi but declined quickly thereafter. The control animals showed no significant rise in mean antibody titre over the duration of the experiment and the titres with the standard antisera to A1 and A9 did not exceed the control values.

The mean serum antibody titres of the infected and control groups, measured in the IHA test, plotted against time are shown in Figure 3.5. Antibody detected in this test increased quickly to a titre of 1 in 48 at 4 pwi then declined sharply afterwards. The mean serum antibody titres of the controls remained almost constant at 1 in 2 throughout the experiment.

The results show that both tests detected antibodies to A2 but some differences are apparent. The IHA test demonstrated maximum titres at 4 wpi while ELISA did not reach a maximum till 8 wpi. This probably reflects the measurement of different antibodies in the tests as the IHA only registers those antibodies reacting with the selected antigens on the red blood cells surface while in the ELISA all antigens bound to the well are available for reaction. It seems probable that both tests are measuring the serotype specific antibodies as the inclusion of heterologous serum in the ELISA did not give high titres and did not register at all in the IHA. In the ELISA the background level of reaction with control sera may be an indication of prior exposure to P.haemolytica which conventional sheep experience.

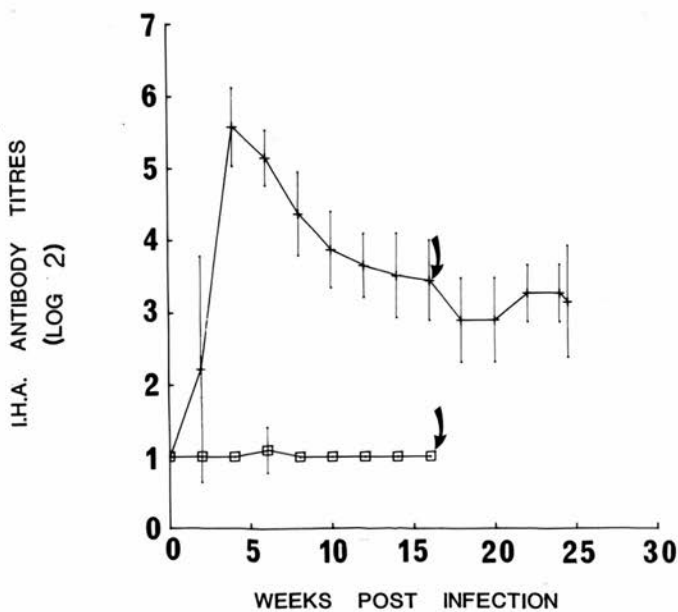
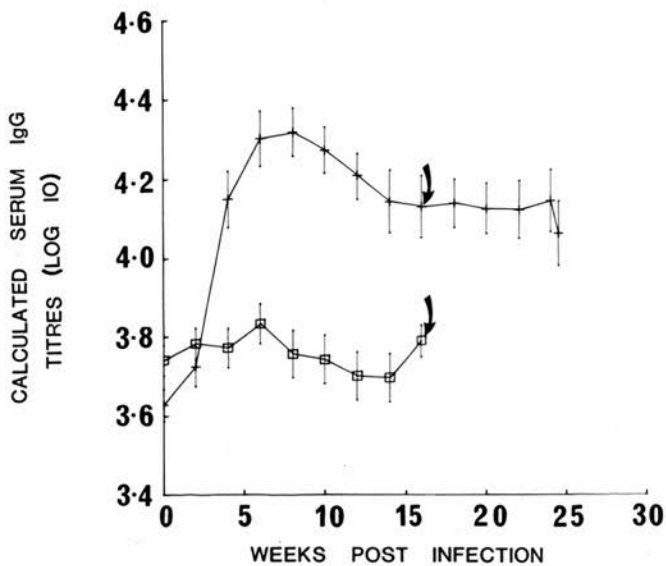


Figure 3.4

Mean calculated serum antibody titres ( $\text{Log}_{10}$ ) against P.haemolytica A2 as detected by ELISA for infected group (+) and control group ( $\square$ ).

Figure 3.5

Mean serum antibody titre ( $\text{Log}_2$ ) against P.haemolytica A2 as detected by IHA for infected group (+) and control group( $\square$ ).



## DISCUSSION

The ELISA technique has been successfully applied to the measurement of antibodies to the antigens of Gram-negative bacteria such as Brucella abortus (Carlsson et al, 1976), Yersinia enterocolitica (Gripenberg et al, 1979), Haemophilus influenzae type B (Crosson et al, 1978) and Bacteroides species (Poxton, 1979). An ELISA to measure antibodies to P.haemolytica SSE antigens was described by Burrells et al (1979) but the complexity of the antigen made the test non-specific: a test set up with one serotype SSE antigen would measure antibody in antiserum to a heterologous serotype (Burrells, personal communication). The ELISA system described here allows the measurement of type-specific antibodies by using PWE antigens in the ELISA.

The cross-reactivity in the ELISA developed by Burrells et al (1979) was demonstrated in the first experiment (section 3.1). When antiserum to A1 was absorbed with heterologous SSE antigens the A405 was slightly reduced in comparison with an unabsorbed control antiserum. Absorption of the antiserum with A1 PWE decreased the A405 by around 50% and when the antiserum was absorbed with both heterologous SSE and PWE then the A405 fell to just above zero indicating that the type specific antibodies had been removed. Reaction of the same treated antisera with All SSE showed that whereas absorption with heterologous SSE's effected a substantial drop in the cross-reaction, absorption with PWE had no effect. This demonstrated a type specificity in PWE which agreed with the previous experiments with IHA and CIE. (Section 1).

Polysaccharide antigens, known to be type specific, have been used in ELISA systems to detect the serotype specific antibodies of such bacteria as Haemophilus influenzae type b (Crosson et al, 1978), Yersinia enterocolitica (Grippenbergh et al, 1979) and Streptococcus pneumoniae (Barrett et al, 1980).

However, problems have been encountered in the binding of polysaccharide antigens to plastic. The antigen binding properties of plastic are little understood but the manufacturers recognise the problems and produce plates by different treatments which are claimed to endow the plastic with different affinities for different antigens (e.g. the 129A and 129B plates from Dynatech). Another approach to the problem has been to use the plastic's known strong affinity for protein antigens. Russell et al (1980) and Barrett et al (1980) both used capture technique to bind pneumococcal antigens and replaced the polysaccharide with antiserum or an IgG fraction in the initial step. Gray (1979) bound poly L-lysine to polysaccharide antigen by a cross linking reagent, cyanuric chloride, and used this hybrid antigen which bound directly to the ELISA plate by virtue of its protein component.

Recently Ito et al (1980) has reported that the inclusion of magnesium ions in the antigen buffer facilitates the binding of rough endotoxin antigens of E.coli and N.gonorrhoeae by some unknown mechanism.

When initial studies showed that A1 PWE was not going to bind easily the possibility of using a sandwich technique with an antibody capture layer was investigated.

Rabbit antibody to P.haemolytica A1 whole cells was used and

in the first attempt whole antiserum was bound to the plate. The weakness of the subsequent colour development was attributed to the competitive binding of the serum components. An IgG fraction of rabbit antiserum is relatively easy to produce on a Sepharose/protein A affinity column and this IgG preparation worked effectively in binding PWE antigen. The serotype specificity obtained with the sandwich ELISA was good when A1 and A9 specificities were investigated and might be applicable to other serotypes.

However, even with PWE a certain amount of cross-reactivity existed. Pre-vaccination SPF lamb sera reacted with A9 PWE. This may indicate a shared determinant with another organism, perhaps in the LPS, as found between Salmonella minnesota, Klebsiella pneumoniae and Proteus morganii (McCabe, 1977) where common LPS core antigen is present.

The results described in Section 3.4 showed that the sensitivity of the ELISA and the use of very small volumes of sera allowed the sequential measurement of antibody from individual mice to be performed. This in turn makes possible the assessment of an individual mouse's response to vaccination as well as the possible link between antibody titre and protection.

The demonstration that A1 PWE can stimulate high titres of circulating antibody shows that mice are responsive to the serotype-specific antigen in this purer form. It also shows that the protection seen on vaccination is not due to some non-specific effect as that described by Evans and Wells (1979b) but due to presence of specific antibody.

Although ten times more SSE than PWE was incorporated into the vaccines the titres obtained against PWE antigen and the protection observed in the two vaccinated groups were comparable, indicating that a substantially smaller dose of purified antigen can elicit the same amount of protective antibody than a larger dose of cruder material. This may have consequences for the formulation of vaccines containing those antigens.

The influence of biological variation between mice was not noticable in the vaccinated groups where the protection and antibody response was 100% but some indication of its effect is seen in the counts obtained in the control group. The livers of two control mice showed low counts after challenge. This could be due to faulty inoculation or to a natural resistance in the mice to challenge. However, it serves to emphasize the need for large numbers in groups to reduce the distortion of results which can occur in this type of experiment.

The solution to the problem of binding A2 PWE was not found in a sandwich ELISA but in a prolonged binding step which means that the time from start to completion of the ELISA is greatly extended.

The comparison of the ELISA for A2 and the corresponding IHA test showed that the antibodies measured in each case seemed to be different as the peak antibody titres in both tests did not correspond. However, the tests both detect specific serotype antibody as shown by the low titres to heterologous sera in both tests.

There is no precise information as to what class of antibody

is responsible for immunity against Pasteurella haemolytica infection, nor is there any defined minimal serum titre value needed to establish immunity. However, a rise in titre against the specific antigen of P.haemolytica A2 as demonstrated in Section 3.7, indicates that the host is experiencing a current infection with A2. This is in line with the findings of Gilmour et al (1982) who showed that P.haemolytica can be recovered from the lungs of sheep with experimentally induced chronic pneumonia up to 11 weeks after infection. There may be some correlation with peak titres of specific antibody at 8 wpi and the clearance of the bacterium soon afterwards.

#### SECTION 4.

##### The analysis of *P.haemolytica* A2 and A6 antigens

#### SECTION 4.1

##### The Serological analysis of *P.haemolytica* A2 and A6 antigens.

The SSE and PWE antigens of A2 were shown to contain the serotype antigens of their respective serotypes by the IHA test. The titres obtained for each antigen preparation are shown in Table 4.1. The SSE and PWE of both serotypes were also run in CIE against homologous antisera raised in sheep and rabbits.

When A2 SSE was run against homologous rabbit antisera at least 9 weakly staining precipitin lines were observed (Fig 4.1a). The pattern obtained with A2 PWE run against rabbit antisera (Fig 4.1b) shows only one very weakly staining precipitin line.

Sheep sera were tried after difficulties in obtaining consistently high-titre rabbit antisera were encountered. The profiles obtained when antisera from SPF lambs were used are shown in Figure 4.2a and 4.2b for SSE and PWE respectively. These show more antigens for SSE than were found with rabbit antisera but again the PWE antigen was only weakly reactive.

When A2 SSE was heated at 115°C for 15 min the profile obtained (Fig 4.2c) showed some changes from that for the untreated extract in that precipitin lines 1 and 2 disappeared.

The SSE and PWE antigens of A6 were also run in CIE against antisera raised in sheep and rabbits. The profiles for A6 SSE when run against rabbit antisera are shown (Fig 4.3). When



10.5ug of A6 SSE were tested against homologous antisera a single precipitin line stretching from the origin almost to the width of the gel was observed (Fig 4.3a). When the loading of the SSE antigen was increased to 50ug, 4 lines were seen in the gel with the strong staining precipitin line noted previously becoming more diffuse and distorted (Fig 4.3b). The incorporation of an intermediate gel containing rabbit antisera to A1 whole cells resulted in the precipitation of the 3 minor antigens of A6 SSE in the intermediate gel (Fig 4.3c). This indicated the cross-reactivity of these antigens whereas the strongly reacting antigen was not impeded. This demonstrated its serotype specificity.

The converse situation, with antiserum to A6 in an intermediate gel and A1 SSE as antigen, did not give a similar pattern, as the antiserum to A6 failed to precipitate any antigens in the immediate gel (Fig 4.4). However, the homologous A1 reaction with SSE at 50ug only gave one strongly staining precipitin line. This was similar in size and shape to that obtained for A6 SSE in Fig 4.3a.

When sheep antisera to A6 was used in CIE there was an increase in the number of antigens precipitated (Fig 4.5a). At least 9 precipitin lines are visible, but again one very strongly staining line can be seen over much of the gel. When antisera to A1 was incorporated in the intermediate gel, 3 antigens were impeded in their progress (Fig 4.5b). These are antigens 2,7 and 9. As with rabbit antisera the converse run with A1 SSE antigen and antisera to A6 in the intermediate gel was carried out. This time two antigens not present in the

homologous A1 reaction were precipitated in the intermediate gel.

The profiles obtained when A6 PWE was run in CIE against homologous rabbit antiserum with and without an intermediate gel containing antiserum to A1 are shown in Figure 4.6. Two precipitin lines can be seen in the homologous reaction with a blank intermediate gel (Fig 4.6a). The major line extends from the origin to beyond the middle of the gel and has a distinct shoulder at the anodic side. The smaller line is present within the area of the major line and is very close to the origin. This smaller precipitin line was impeded in the intermediate gel when antiserum to A1 was incorporated into this gel.

The CIE profiles for A2 and A6 SSE are distinctly different from that obtained for A1 SSE as they demonstrate a complex mixture of antigens not seen in the A1 studies. However, with PWE antigens the results with A6 were comparable to A1 in that two antigens were present one of which was cross-reactive with antiserum to a heterologous serotype. The A2 PWE antigen was less antigenic than either A1 or A6 and appeared to consist of one weakly staining precipitin line in CIE.

Table 4.1

The IHA titres of SSE and PWE antigens of P.haemolytica A2 and A6 when tested against homologous antisera.

SEROTYPE	ANTIGEN	IHA titre
A2	SSE (2.5mg ml <sup>-1</sup> )	1000
	PWE (1mg ml <sup>-1</sup> )	1000
A6	SSE (2.5mg ml <sup>-1</sup> )	1000
	PWE (1mg ml <sup>-1</sup> )	10,000

Figure 4.1

Crossed immunoelectrophoresis (CIE) of a)A2 SSE(40ug) and b)A2 PWE(10ug) run against 130ul of rabbit antisera to A2 whole cells.

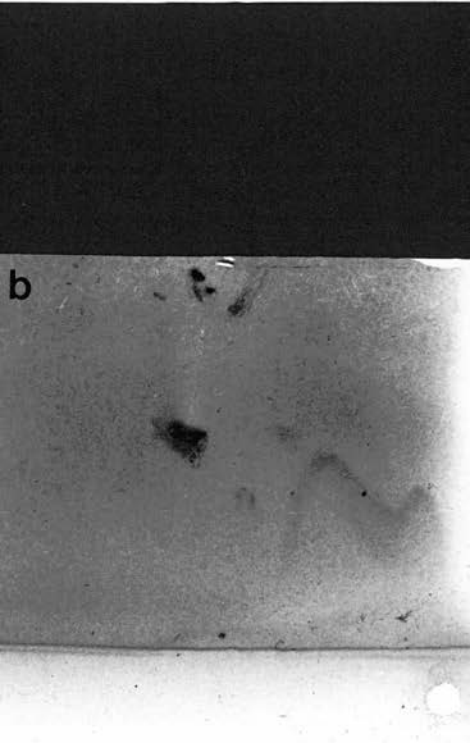
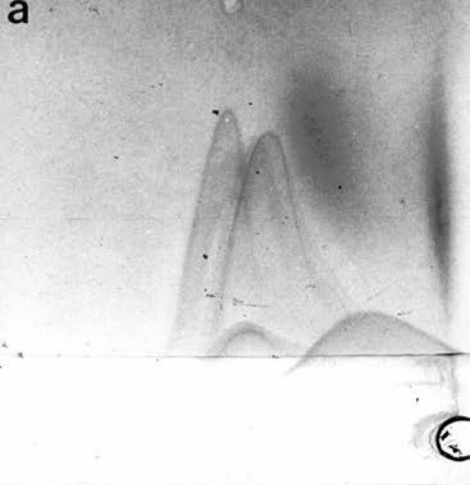


Figure 4.2

CIE of a)A2 SSE (40ug) and b)A2 PWE (10ug) and c)A2 SSE (40ug) heated at 115°C run against 130ul of SPF lamb antisera to A2 whole cells.

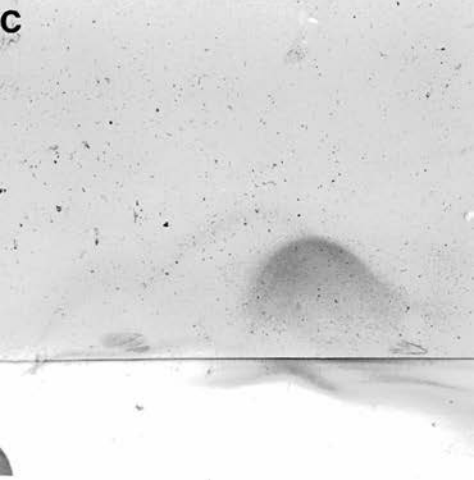
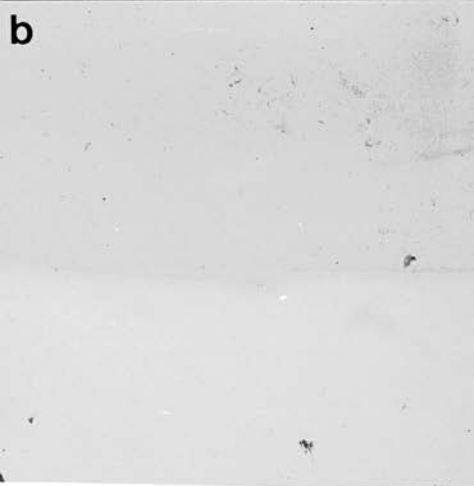
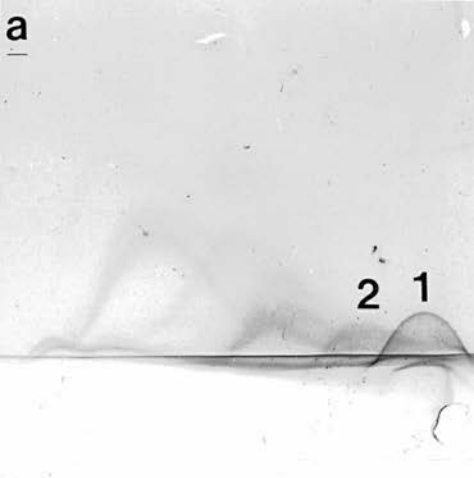


Figure 4.3

CIE of a)A6 SSE (12.5ug) b)A6 SSE (50ug) run against 130ul of rabbit antisera to A6 whole cells and c)the profile of A6 SSE (50ug)run against 100ul of antisera to A6 and an intermediate gel containing 30ul of antiserum to A1 cells.



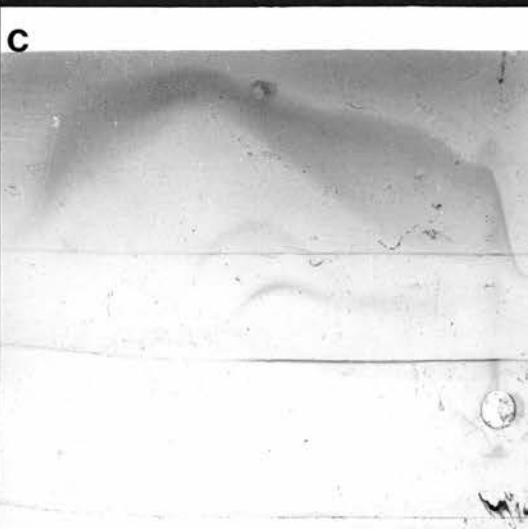
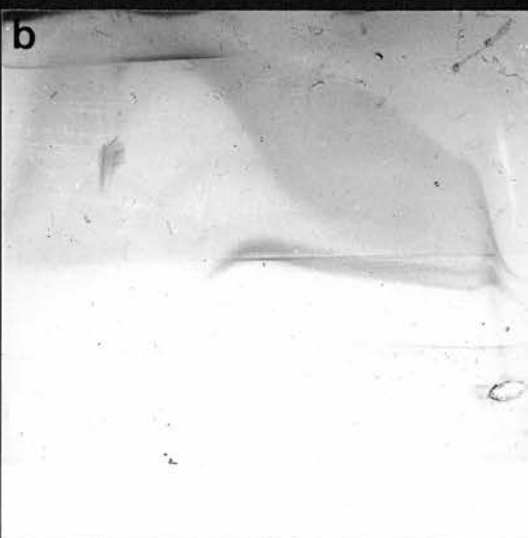
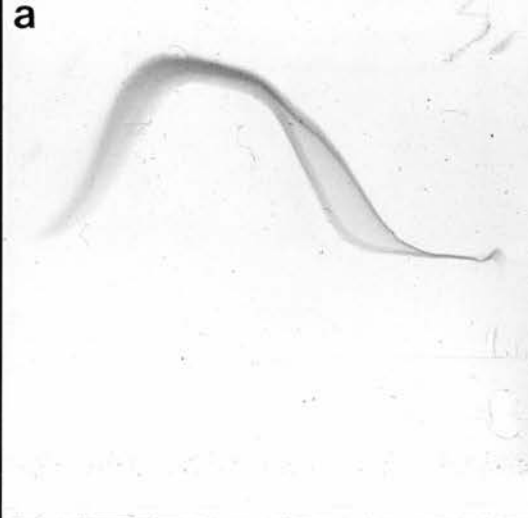


Figure 4.4

CIE of A1 SSE (50ug) run into an agarose gel containing 130ul of homologous rabbit antiserum with an intermediate gel containing 30ul of rabbit antiserum to A6 whole cells.

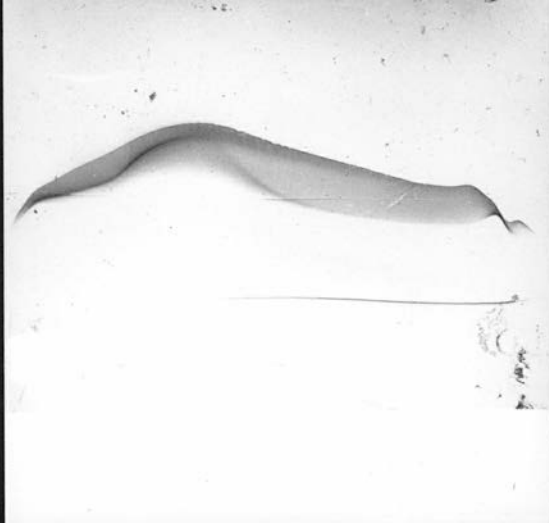


Figure 4.5

CIE of A6 SSE(12.5ug) run against 100ul of homologous sheep antiserum to A6 with a)blank intermediate gel and b)an intermediate gel containing 30ul sheep antiserum to A1. c) CIE of A1 SSE (50ug) run against 100ul of homologous sheep antiserum to A1 with an intermediate gel containing 30ul sheep antiserum to A6.

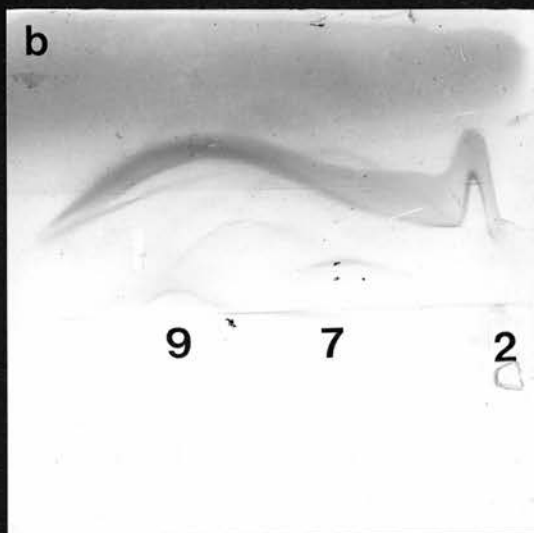
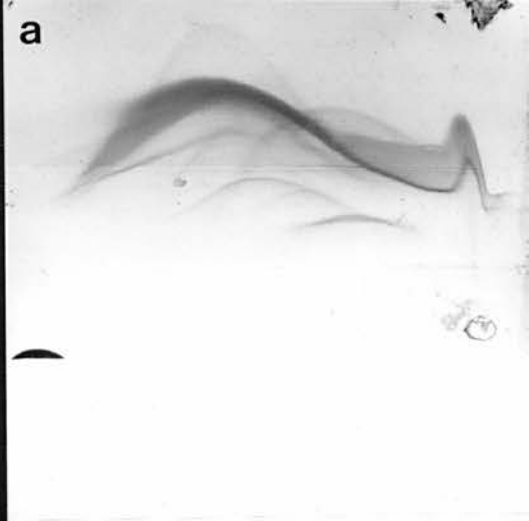


Figure 4.6

CIE of A6 PWE (10ug) run against 100ul homologous rabbit antiserum a)with a blank intermediate gel and b)with an intermediate gel containing 30ul of rabbit antiserum to A1

**a**



**b**



## SECTION 4.2

### Further characterisation of the PWE antigens of *P.haemolytica*

#### A2 and A6.

The serotype specificity seen in A1 PWE is also present in A6 PWE and may also be present in A2 PWE. This suggests that the basis of serotype differences might be discerned by the chemical analyses already used on serotype A1 when applied to A2 and A6.

The results of chemical analyses on A2 and A6 PWE are shown in Table 4.2 and for comparison the corresponding results for A1 PWE are included. The results indicate that A1 and A6 are similar in composition with some slight quantitative differences. A2 PWE has very little carbohydrate (2%) and this may explain the failure to measure heptose content.

*P.haemolytica* A2 was subjected to partial hydrolysis treatment with 1% acetic acid and the supernate from the hydrolysis was chromatographed on Sephadex G50 as already described for A1 (Section 2.1). Fractions collected from the column were assayed for total carbohydrate and the results are shown graphically in Figure 4.7.

One major peak was observed between fractions 31 and 37. No antigen was measurable by FRIE in the A2 PWE after partial hydrolysis. The PWE's of A2 and A6 were analysed by GC and copies of both traces are shown (Fig 4.8). These show that both PWE's are very similar to each other and also similar to A1 (Section 2.1). Both contain mannose, galactose, glucose, two heptoses, glucosamine and galactosamine in the molar ratios,



1.3; 0.7; 1.0; 0.1; 0.2; 0.8; 0.4 and 0.7; 0.4; 1.0; 0.1; 0.09; 0.3; 0.1 for A2 and A6 respectively.

The fractions comprising the carbohydrate peak from the G50 separation of A2 PWE partial hydrolysate (fractions 31-39) were pooled and analysed by G.C. A copy of the trace is shown in Figure 4.8 and the presence of glucose, galactose and two heptoses is clearly indicated. This is similar to the finding for A1 peak II (Section 2.1).

Although quantitative differences are obviously present between A2, A6 and A1 it would appear that they all have a similar monosaccharide composition. The similar behaviour of the partial hydrolysate of A2 PWE and A1 PWE in FRIE and on column chromatography reinforces the similarity in structure that must exist between the serotypes.

Table 4.2

Composition of PWE's prepared from A1, A2 and A6 (values are expressed as percent of total dry weight).

COMPONENT	SEROTYPE		
	A1	A2	A6
Phosphorus	1	4.4	2.8
Protein	6.3	1.6	0.4
Carbohydrate	14.8	2	12.4
Fatty Acid	10.8	ND	5.8
Heptose	6.6	0	2.4
KDO	0.6	0.4	0.01

ND = Not Done

Figure 4.7

Elution profile of the aqueous portion of partially hydrolysed A2 PWE on Sephadex G50. Fractions (50 X 3ml) were eluted with water and assayed for carbohydrate by the phenol sulphuric acid method (Dubois et al 1956).

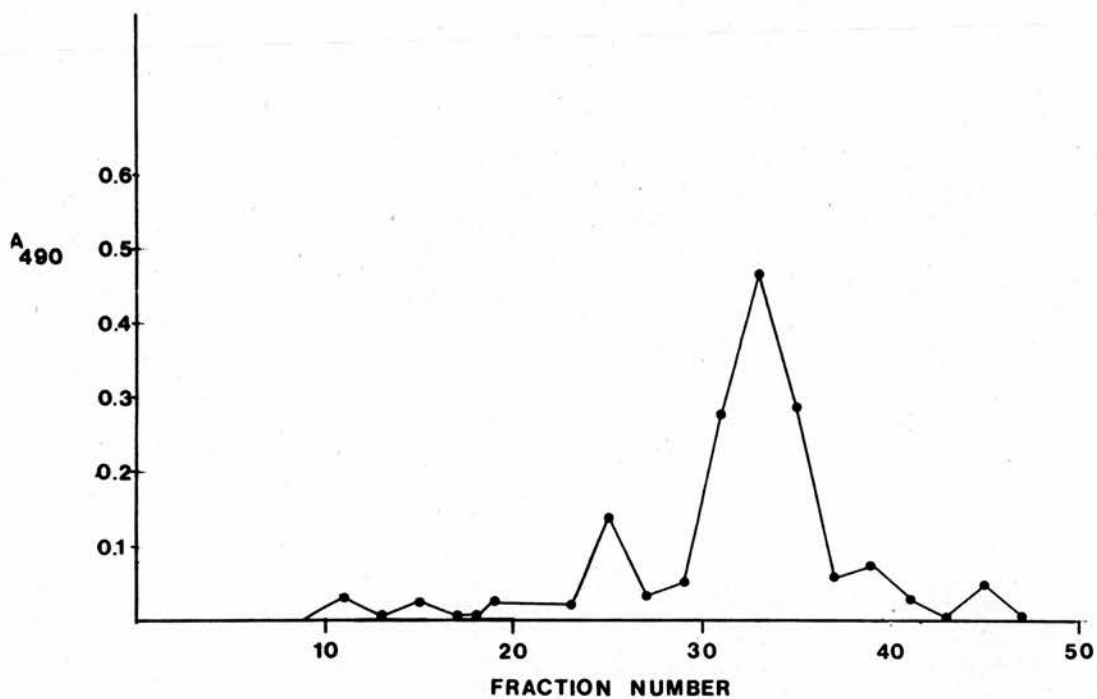
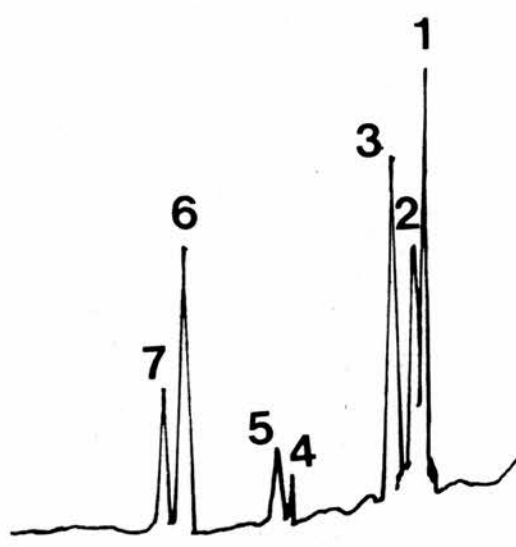


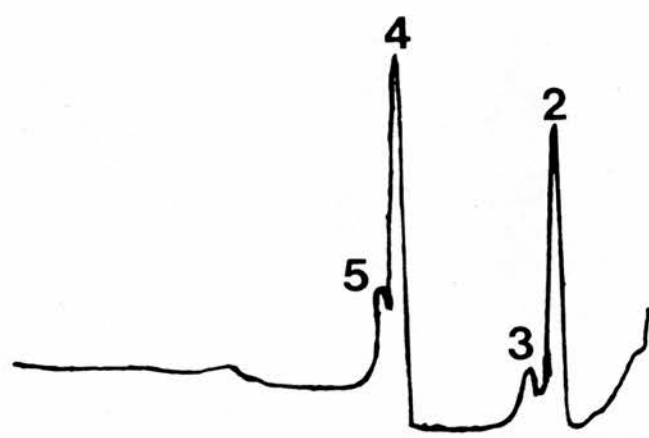
Figure 4.8

GC of alditol acetate derivatives of acid hydrolysates of P.haemolytica A2 and A6 PWE preparations on columns of OV225 for the detection of sugars. a)untreated A2 PWE; b)untreated A6 PWE; c)The major sephadex G50 peak from partially hydrolysed A2 PWE. The sugars present are identified as follows: 1, mannose; 2, galactose; 3, glucose; 4, heptose 1; 5, heptose 2; 6 glucosamine; 7, galactosamine.

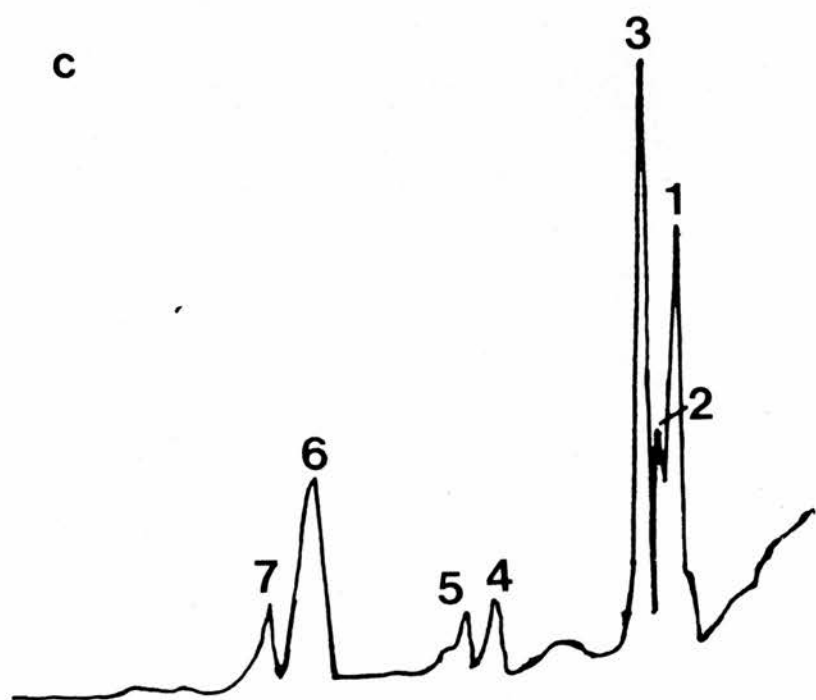
a



b



c



### SECTION 4.3

The immunogenicity of *P.haemolytica* A2 antigens in mice.

*P.haemolytica* A2 is probably the most important serotype in sheep pasteurellosis and protection against this serotype is therefore crucial to the success of any vaccine. However, the demonstration of protection by A2 SSE in sheep (Gilmour et al, 1983) has not been similarly demonstrated in mice and Evans (1979) was unable to show protection in her studies in mice.

This failure may have been due to the wrong presentation of the antigens and the following experiments were designed to investigate the potency of different preparations of A2 antigens in protecting against challenge.

In all vaccines except for that containing live cells the antigens were absorbed onto aluminium hydroxide before emulsification in oil as described in Materials and Methods.

Vaccines incorporating the SSE, EDTA and PWE antigens of A2 were tested in mice as described in Materials and Methods and the results are shown in Table 4.3. No protection was evident with EDTA or PWE preparation although A2 SSE at a concentration of 280ug dry weight per dose gave significant protection ( $P < .001$ ).

A second experiment involved the inoculation of live A2 organisms. One group of 40 mice was challenged twice with an LD<sub>50</sub> of A2, the survivors were kept and 10 of these re-challenged. A second group was given two s.c. doses of live A2 organisms ( $1 \times 10^5$  per dose) on the same schedule as that of the other vaccines. These two groups were challenged along with

a third group vaccinated with A2 SSE at a concentration of 280ug per dose. The results (Table 4.4) indicated that the LD50 survivors are protected against challenge ( $P \leq .001$ ) while the other two groups are not.



Table 4.3

Counts<sup>1</sup> of P.haemolytica A1 in the livers of unvaccinated control mice and mice vaccinated with A2 SSE, EDTA or PWE challenged with P.haemolytica A2 (strain S51)<sup>2</sup>

VACCINE	VACCINE DOSE	MEAN COUNT IN LIVER After 6h	P
A2 SSE	(284ug)	4.6±1.4	<.001
A2 EDTA	(50ug)	6.2±2.0	NS
A2 PWE	(50ug)	6.8±0.8	NS
Not Vaccinated	-	6.9±0.9	

1. Counts = cfu expressed as Log<sub>10</sub> values

2. Challenge dose ( $3.15 \times 10^6$  cfu)

Table 4.4

Counts<sup>1</sup> of P.haemolytica A2 in the livers of unvaccinated control mice, mice vaccinated with A2 SSE or live A2 organisms and mice surviving LD<sub>50</sub> challenge. Challenge with P.haemolytica A2 (strain FA2)<sup>2</sup>.

GROUP	Mean count in liver after 6h	P
A2 SSE Vaccine (284ug per dose)	5.83±1.2	NS
A2 live cells (1x10 <sup>5</sup> cfu per dose)	5.75±1.42	NS
LD <sub>50</sub> survivors	3.04±0.99	<.001

1. Count - cfu expressed as log<sub>10</sub> values

2. Challenge dose = 1.75 X 10<sup>5</sup> cfu

SECTION 4.4The immunogenicity of A6 antigens in mice.

The similarities noted between A6 and A1 antigens in the immunochemical analysis results suggested that there may also be similarities in the antigens required to stimulate protection in mice. To test this hypothesis mice were vaccinated with the SSE and PWE antigens of A6 and A1. The possibility of cross-protection between PWE antigens was investigated by heterologous challenge.

The antigens were made up in CFA and the dosages are given with the results in Table 4.5.

The PWE antigens of both A1 and A6 conferred significant protection against homologous challenge ( $P=.01$ ) and PWE of A6 also stimulated protection against heterologous challenge ( $P=.05$ ). The degree of protection with PWE seems to be less than that given by the SSE's of both ( $P=.001$ ) against homologous challenge.

Table 4.5

Viable counts of P.haemolytica A1 or A6 in the livers of control mice and mice vaccinated with SSE or PWE from P.haemolytica A1 or A6.

VACCINE	CHALLENGE (DOSE)	VIABLE COUNT ( $\text{LOG}_{10}$ )	
		CONTROL	VACCINATED
A1 SSE	A1 ( $1.3 \times 10^6$ cfu ml <sup>-1</sup> )	6.68±1.7	3.4±0.9 <sup>1</sup>
A1 PWE	A1 ( $1.3 \times 10^6$ cfu ml <sup>-1</sup> )	6.68±1.7	4.9±1.8 <sup>2</sup>
A6 PWE	A1 ( $1.3 \times 10^6$ cfu ml <sup>-1</sup> )	6.68±1.7	5.1±1.9 <sup>3</sup>
A6 SSE	A6 ( $2.9 \times 10^5$ cfu ml <sup>-1</sup> )	7.42±0.4	2.8±0.3 <sup>1</sup>
A6 PWE	A6 ( $2.9 \times 10^5$ cfu ml <sup>-1</sup> )	7.42±0.4	4.5±1.7 <sup>2</sup>
A1 PWE	A6 ( $2.9 \times 10^5$ cfu ml <sup>-1</sup> )	7.42±0.4	6.7±0.9 <sup>4</sup>

1 = Significant at .001 level (Mann-Whitney ranking test)

2 = Significant at .01 level

3 = Significant at .05 level

4 = Not Significant

### DISCUSSION.

The results of CIE studies on P.haemolytica A2 and A6 SSE preparations showed some differences from those obtained from A1 SSE. The most obvious was the presence of substantially more precipitin arcs with A2 and A6 SSE, especially with sheep antisera. In contrast to the findings with A1 SSE, where a major polysaccharide antigen predominated, the A2 SSE CIE profile showed no such antigen. Heat treatment of A2 SSE altered the profile of the 9 precipitin arcs indicating heat lability and the probability of protein composition for some of these antigens. With A6 however, the predominant antigen in the SSE was also the major arc in the PWE and the serotype specific antigen indicating that a similarity with the serotype antigen of A1 existed and that they are both polysaccharide in nature. The PWE of A2 was only weakly antigenic although it did show a mobility and an appearance in gels similar to the slow moving double humped precipitin line seen with A1 PWE (Fig. 1.1d). This is probably not the serotype antigen as it was precipitated by antisera to A9 indicating that it was not serotype specific (Fig. 1.3). It may be that the serotype specific antigen of A2 which has been shown to be present in PWE by IHA and ELISA (section 3.6) is not precipitated in gels.

The comparison of the CIE results from the 3 serotypes A1, A2 and A6 suggests that the antigenic composition of the A1 SSE is simpler than that of A2 or A6 SSE and consists of only 3 or 4 different antigens. However, there may be antibody/antigen reactions which are obscured by the strong reaction of the serotype antigens as antigens not seen in the A1 SSE homologous

reaction were precipitated by antiserum to A6. The observation that the antiserum to A1 precipitates antigens in the A6 SSE showed that antibodies to antigens other than the serotype antigen were present in the antiserum. This would seem to imply that the serotype antigen of A1 obscures the reaction of other antigens in CIE gels.

The differences seen in the gel profiles when sheep antisera were used in place of rabbit antisera are possibly related to the host specificity of P.haemolytica and the way that different species perceive the chemical configuration of the antigens.

It is possible that rabbits and sheep respond to different antigens, for example P.haemolytica A2 cells may contain antigens which mimic sheep cellular antigens thus avoiding the immune response of sheep. This has been shown to occur in the polysaccharide K-antigens of E.coli K1 and N.meningitidis group B where those antigens mimic antigens in human brain tissue (Finne et al, 1983).

However, studies with A2 antigens indicated that it is difficult to immunise mice against this organism. The result with A2 SSE (Table 4.3) was not reproducible (Table 4.4) and the variation seen in liver counts with this antigen suggests that although some immunity is given it is not sufficient to protect fully against challenge. The only effective procedure was to challenge LD<sub>50</sub> survivors and here the liver counts were consistently low although it is possible that the protection only reflects innate resistance in certain mice. However the initial group of mice challenged contained 40 mice of which 18 survived an LD<sub>50</sub> challenge and 10 of these a subsequent

challenge in the mouse model. If this figure is consistent then approximately 47% of all mice challenged should demonstrate protection. This does not occur at this frequency with unvaccinated control mice for A2 challenges or in other serotype challenges, although frequently one mouse out of ten does not succumb to challenge. Another possible explanation for the apparent protection is that the i.p. challenge has stimulated a non-specific protection.

It is interesting that a high titre antibody response in sheep to A2 antigens is found only after infection as shown in Section 3.7. High IHA and ELISA titres of antibodies to specific serotype antigens of A2 were only obtained after infection of sheep with live A2. In SPF lambs the highest antibody responses also occur after challenge with live organisms (Gilmour, personal communication).

It is known that protection against facultative intracellular bacteria such as Salmonella and Brucella species is generally better when living, attenuated vaccines are given rather than killed organisms (Collins and Campbell, 1982) and the same authors suggest that perhaps P.haemolytica is a facultative intracellular bacteria. It is suggested that the improved immunity associated with live vaccines arises from the continual production of antigens, some of which may be unique "metabolic antigens" which react with T lymphocytes. These in turn produce lymphocytes to activate the macrophages which destroy the bacteria which induced the initial response.

The possibility that killed antigens were not the same as when in their native state was suggested by Gilmour et al (1983)

to explain the need for both SSE and heat killed organisms in A2 vaccines. These authors proposed that the presentation and quantity of the antigens were important in the induction of immunity.

The resistance of mice vaccinated with A6 PWE to homologous challenge is comparable to the A1 finding (section 1.2) and confirms that the PWE contains an antigen which confers protection on some serotypes. As discussed earlier (section 1) this is in agreement with the findings of Biberstein and Thompson (1965) who concluded from studies on mouse protection that the "capsular" (i.e. the serotype antigen) was more important than the somatic antigens in stimulating protection.

The results of the mouse protection studies with A1 and A6 antigens also demonstrate the possible cross-protective properties of P.haemolytica PWE antigens. The cross-protection observed was a one-way cross with A6 PWE conferring protection against A1 challenge. Evans (1979) did not observe cross-protection in mice vaccinated with SSE antigens where protection was serotype specific. The cross-protection evident in A6 PWE vaccinated mice may be explained by the presence of antigens in A6 PWE which are important in the protection of both A6 and A1, while A1 PWE lacks some of these antigens. Gilmour (personal communication) has noted that A1 and A6 sometimes cross-react in the IHA test which is normally serotype-specific. The antigens important in cross-protection may normally be hidden by cell surface components, even in SSE, and are only exposed when isolated in the PWE. McCabe et al (1977) reported that a rough RE mutant of Salmonella minnesota, which possessed



only KDO and Lipid A components of LPS, conferred more immunity against challenge with Klebsiella pneumoniae and Proteus morganii than did the parent strain. However, the homologous protection with the mutant strain was less than that observed in the parent strain. The authors indicated that in addition to possible disclosure of previously hidden antigens the mutation to RE may also have produced other component changes in the outer membrane of the bacterium.

Cross-protection was also observed in section 5 when the SSE antigens of some group II IHA negative isolates of P.haemolytica were shown to cross-protect against group I isolates (Section 5.4). However this cross-protection did not extend to the IHA positive serotypes A1 and A2 so their potential as non-specific immunisation agents is probably limited to the IHA negative strains.

Mukkur (1977) demonstrated cross-protection between P.haemolytica A1 and P.multocida type A when extracts prepared by potassium thiocyanate treatment of A1 cells were used as vaccines. It may be that other serotypes of P.haemolytica share antigens with P.multocida and this could offer a means of obtaining a common protective antigen.

The chemical analyses showed that the PWE antigens of A1 and A6 were very similar and although the sugar composition of A2 PWE's was similar to the other two the yield of carbohydrate was low (approx 2%) compared to A1 (14.8%) or A6 (12.4%). The sugar composition of the PWE's were remarkably similar and as found with A1, two distinct heptoses are present in A6 and A2. The similarity between the serotypes was further emphasised by the

composition of the pooled fractions from the Sephadex G50 chromatography of A2 PWE. This showed that A2 and A1 PWE had similar compositions. This finding suggests that the differences seen in CIE are probably due to configurational changes in the carbohydrate structure and are not due to the presence of completely different components. A similar finding was described by Hatten and Brodeur (1978). They found that biotypes of Brucella abortus S19 strain could not be differentiated on their elution from chromatography columns, or chemical composition but could be differentiated in their antigenicity on immunodiffusion studies.

SECTION 5The surface antigens of some IHA negative isolates of  
P.haemolytica.

Up to the present time all isolates of P.haemolytica which do not serotype with the 15 prototype antisera have been assigned into a single group. The importance of this group is indicated by the finding that approximately 11% of all isolates referred to the MRI in the years 1978 to 1982 belonged to this group (Fraser et al, 1982a). This is double the percentage from a survey carried out a few years previously (Thompson et al, 1977). Biberstein et al (1960) and Aarsleff et al (1970) used type specific antisera and agglutination tests and did not discover any groupings within their IHA negative isolates; autoclaved cells from these isolates either reacted with more than one antisera or failed to react at all. These workers have speculated that the IHA negative isolates are members of the 15 IHA positive serotypes and this absence of reactivity in the IHA test results from lack of soluble serotype antigen on the cell surface.

However, recent work by Frank (1980) on ten IHA negative isolates from cattle has shown that those isolates could be grouped into 3 serotypes on the basis of their reactions in a rapid plate agglutination (RPA) test. Frank proposed that these serotypes are distinct from the established IHA positive serotypes and because of this that all IHA negative isolates should be examined for specific serotype antigens by the RPA

test.

As the MRI provides a P.haemolytica serotyping service for the U.K. a large number of IHA negative isolates have been identified and stored. The serological techniques used in the analyses of A1 antigens were applied to the investigation of the antigenicity of some of these IHA negative isolates and the following experiments were carried out.

### SECTION 5.1

The analysis of the surface antigens of IHA negative isolates by rapid plate agglutination), agarose gel diffusion and immunoelectrophoresis.

Thirty one isolates of P.haemolytica which were referred to the MRI for serotyping but which failed to react in the IHA test were recovered from storage at  $-70^{\circ}\text{C}$ . They were confirmed as P.haemolytica by colonial morphology and on the basis of the biochemical characteristics described by Biberstein (1978). They were biotyped by their ability to ferment either arabinose or trehalose (Biberstein, 1978). All IHA negative isolates tested belonged to the A biotype.

Six isolates, UT5, 6, 7, 10, 18 and 19 (all ovine isolates) were selected from the collection and antiserum to each individual isolate was raised in rabbits by the hyperimmunisation schedule given in Materials and Methods. The cells of these six isolates were extracted with sodium salicylate and this was further extracted with hot phenol-water as described in Materials and Methods. The six isolates were examined initially in the RPA test and subsequently with AGD and CIE methods as described in Materials and Methods.

The six isolates were examined in the RPA with rabbit antiserum and the results are shown in Table 5.1. These indicated that two distinct serogroups were present, one group containing UT5, 7, 18 and 19 and the second UT6 and 10. However, when representative antisera from these two groups (UT6 and UT18) were absorbed with each other's cells and used to examine the remaining isolates in the collection (Table 5.2) it

was found that 7 isolates were agglutinated with antiserum to UT6, 6 with antiserum to UT18, 3 isolates were agglutinated with both antisera, one isolate autoagglutinated and the remaining 14 isolates were not agglutinated by either antiserum.

When the SSE antigens of the initial six isolates were tested in AGD against homologous antiserum and antisera to the other 5 isolates the results confirmed the existence of the two groups found in the RPA test. The patterns obtained when the six SSE preparations were tested against antisera to UT6 or UT18 were representative of the two groups and are shown (Fig 5.1.) Two distinct precipitin lines were present in reactions between antiserum and antigens of isolates in the same group. There was no reaction visible between antiserum and antigens from different groups. When PWE antigens were tested with the group representative antisera, the two groups were again easily distinguished although only one precipitin line was obtained in the homologous reaction. (Fig 5.2).

The results indicated that antigens important in distinguishing serogroups of IHA negative isolates could be precipitated in gels with antiserum and that they were contained in phenol-water extracts.

The pattern obtained when the SSE antigens of UT18 were run against homologous antiserum in CIE is shown in Figure 5.3a. Two antigens are clearly precipitated in the gel. The larger of the two precipitin line stretches back to the origin and runs just behind the smaller. Within the larger precipitin arc there appears to be more than one antigen. When UT6 SSE antigens were similarly examined one major precipitin line was observed with a

possible second line present just in front at the anodic side (Fig 5.3b). The specificity of these antigens for the group is seen by the precipitation of UT18 SSE in an intermediate gel containing antiserum to UT7 (Fig 5.3c) but not one containing antiserum to UT6 (Fig 5.3d). The AGD result demonstrated that the PWE contained the group-specific antigens and this was confirmed when UT6 and UT18 PWE antigens were run in CIE. The patterns obtained when UT6 and UT18 were tested with homologous antisera are shown in Figure 5.4a and d respectively. Two distinct antigens were precipitated in each case, and with UT18 PWE, these were very similar to those seen in the reaction with the SSE of this strain (Fig. 5.3a). The incorporation of heterologous antisera into intermediate gels did not impede the antigens of UT6 or 18 and these precipitated in the homologous gel (Fig. 5.4b and e). However, when UT6 PWE antigens were run through an intermediate gel containing antiserum to UT10 the antigens were precipitated in the intermediate gel (Fig. 5.4c). Similar reactions occurred when UT18 PWE antigens were run through intermediate gels containing antisera to one of the following; UT5, 7 or 19. The reaction for UT5 is shown (Fig. 5.4f). The group-specificity of the antigens is therefore confirmed by these results.

The cross-reactivity which has already been shown to exist between the SSE's of different IHA positive serotypes (Section 4.1) was also shown to be present between an IHA positive serotype and an IHA negative isolate. The SSE from UT5 was run in CIE with an intermediate gel containing antiserum to A2 (Fig. 5.5). The gel shows that 4 distinct antigens react with

antisera to A2 but not the group-specific antigen which precipitates in the main gel containing antiserum to UT5.

When the PWE of the two groups were tested on AGD against antisera to the IHA positive serotypes only one reacted. Antiserum to All reacted strongly and specifically with the antigens of UT6 and 10. This reaction was one-way and antiserum to UT6, absorbed with UT18 cells, did not react with All antigens in IHA, AGD or CIE. The reaction of antiserum to All with UT6, UT10 and All antigens in AGD are shown in Figure 5.6a. The homologous antigen is different from the heterologous antigens as seen by the non-identity of the precipitin areas. In addition, there is no observable reaction between antiserum to UT6 and All antigen (Fig. 5.6b).

It would appear that the antiserum to All recognises an antigen in the IHA negative group which is not present in the homologous All extracts.

These results have demonstrated that group specific antigens were present, presumably on the surface of the cells of IHA negative isolates. The RPA was not reliable enough to group all the isolates but AGD and CIE tests have shown that the group specific antigens can be precipitated with specific homologous antiserum. Furthermore the presence of these antigens in phenol-water extracts strongly suggests that they are polysaccharide in nature.



Table 5.1

The RPA reations of six IHA negative isolates of P.haemolytica.

CELLS	RPA REACTION WITH THE FOLLOWING ANTISERA					
	UT6	UT10	UT5	UT7	UT18	UT19
UT6	+	+	-	-	-	-
UT10	+	+	-	-	-	-
UT5	-	-	+	+	+	+
UT7	-	-	+	+	+	+
UT18	-	-	+	+	+	+
UT19	-	-	+	+	+	+

+ = Agglutination

- = No agglutination

Table 5.2

The response of IHA negative isolates when tested with antisera to UT6 and UT18 in the RPA test.

ISOLATE	AGGLUTINATION WITH ANTISERA TO		ISOLATE	AGGLUTINATION WITH ANTISERA TO	
	UT6	UT18		UT6	UT18
UT2	-	-	28	-	-
3	-	-	29	+	-
5	-	+	30	+	+
6	+	-	31	+	-
7	-	+	33	-	-
8	-	+	34	+	-
10	+	-	35	-	-
18	-	+	36	-	-
19	-	+	37	-	-
21	-	-	38	-	-
22	+	+	39	-	+
23	+	+	40	-	-
24	-	-	42	-	-
25	-	-	43	+	-
26	-	-	44	+	-
27	AA	AA			

+ = Agglutination

- = No agglutination

AA = Auto agglutination

Figure 5.1

The agarose gel precipitin patterns obtained with a)the antiserum to UT6 and b)the antiserum to UT18 diffused against the SSE antigens (40ug) of 1)UT5, 2)UT6, 3)UT7, 4)UT10, 5)UT18 an 6)UT19. The gel was stained with Coomassie blue.

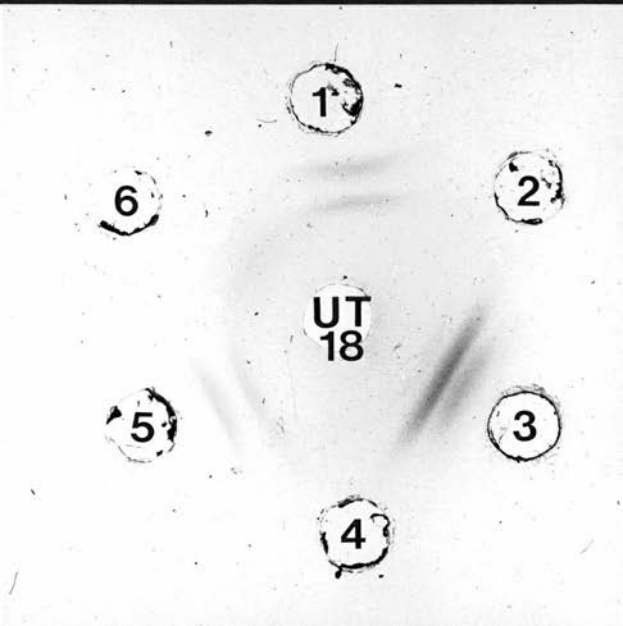
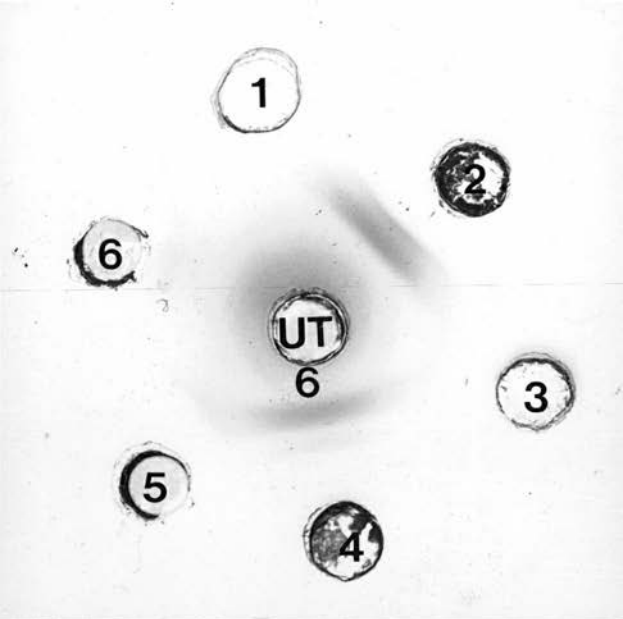


Figure 5.2

The agarose gel diffusion patterns obtained with a)the antiserum to UT6 and b)the antiserum to UT18 diffused against the PWE antigens (10ug) of 1)UT5, 2)UT6, 3)UT7, 4)UT10, 5)UT18 and 6)UT19. The gel was stained with Coomassie blue.

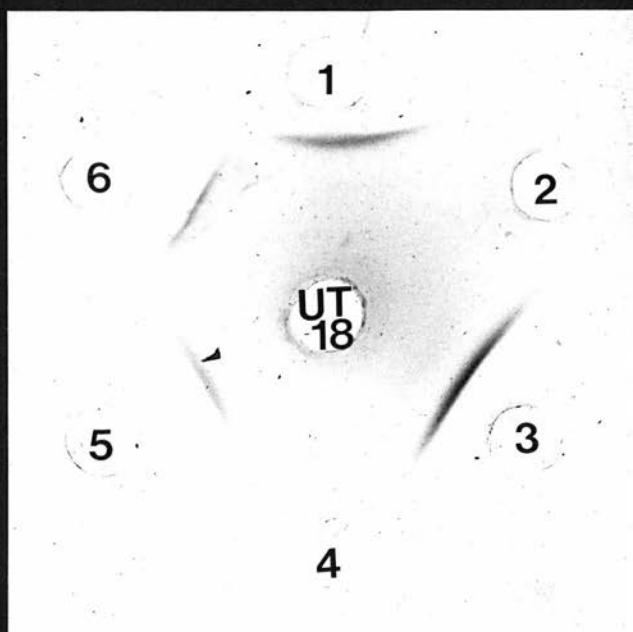
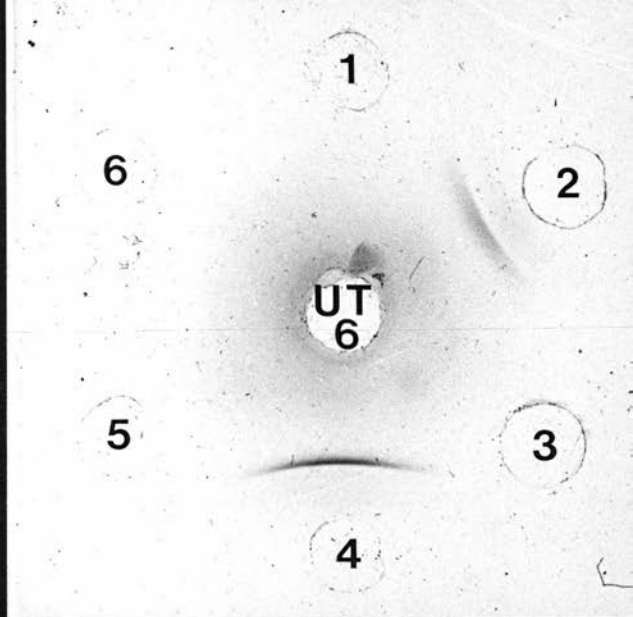


Figure 5.3

Crossed immunoelectrophoresis of UT18 SSE (40ug) run against 100ul of homologous antiserum with an intermediate gel containing a)no antiserum c)30ul of antiserum to UT7 and d)30ul of antiserum to UT6. The SSE of UT6 (40ug) was run against 100ul of homologous antiserum with no antiserum in the intermediate gel (b). The gel was stained with Coomassie blue.

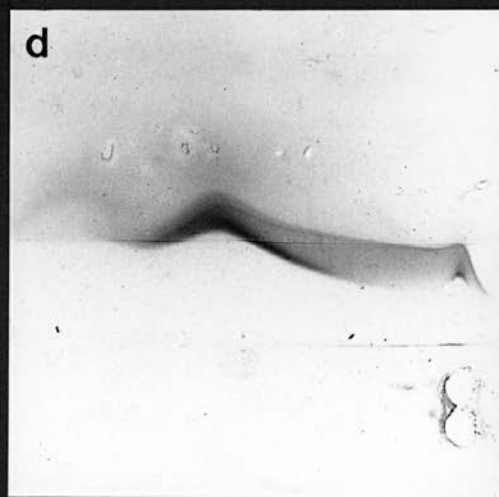
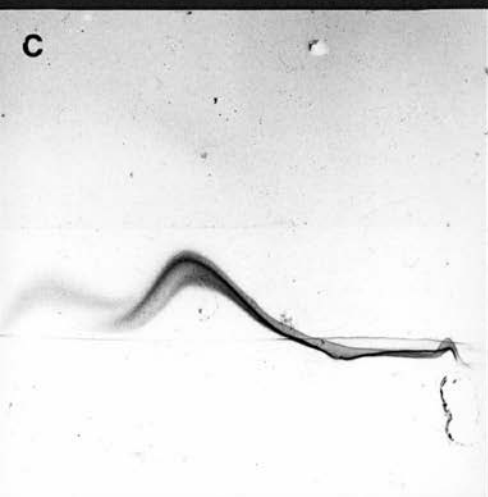
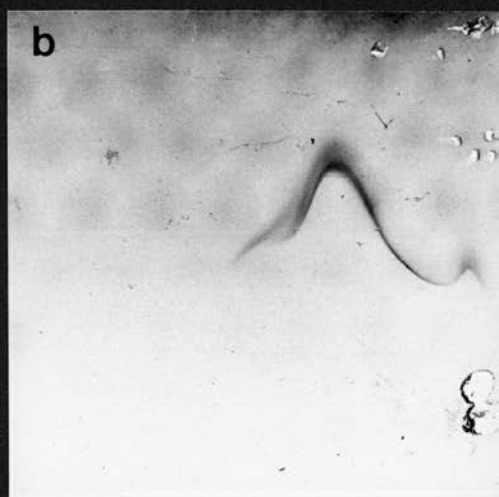
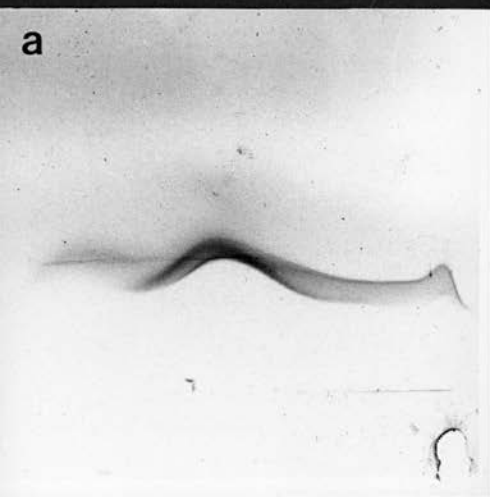




Figure 5.4

Crossed immunoelectrophoresis of UT6 PWE (10ug) against 100ul of antiserum to UT6 with the incorporation of an intermediate gel containing a)no antiserum, b)antiserum to UT18, c)antiserum to UT10. UT18 PWE (10ug) run against 100ul of antiserum to UT18 with the incorporation of an intermediate gel containing d)no antiserum, e)antiserum (30ul) to UT10, f)antiserum (30ul) to UT5. The boundaries of the intermediate gels are shown by the dashed lines. The gel was stained with Coomassie blue.

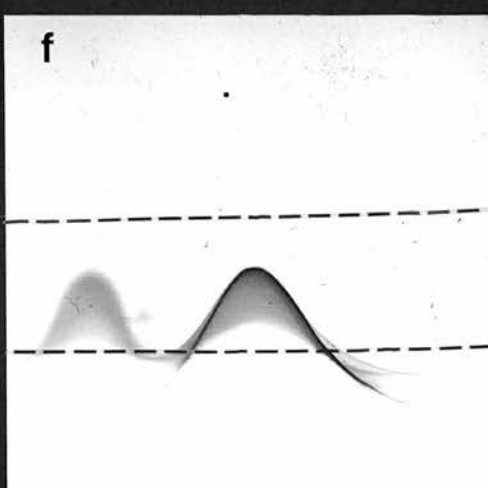
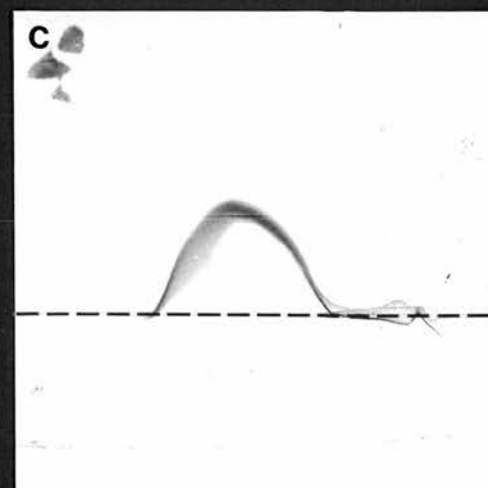
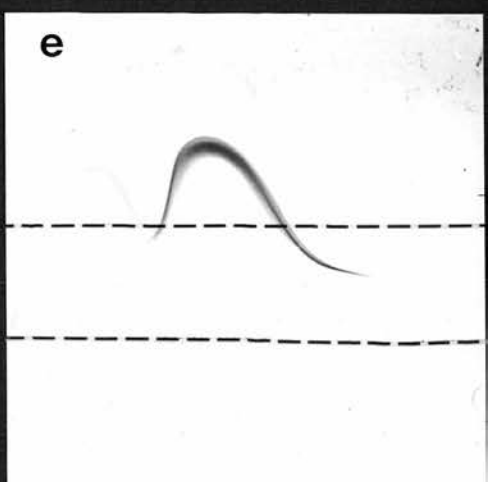
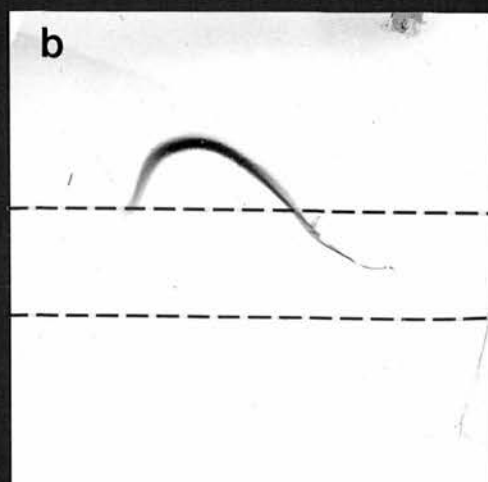
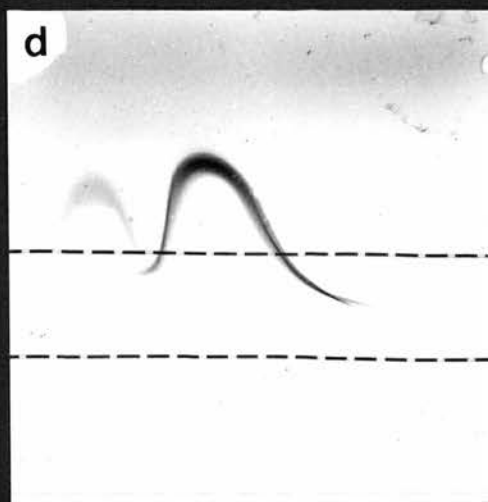
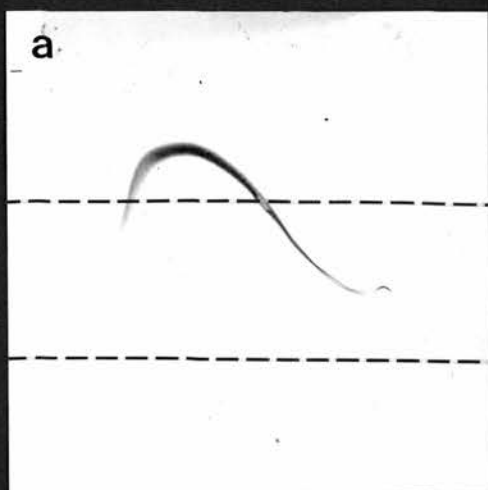


Figure 5.5

Crossed immunoelectrophoresis of UT5 SSE (40ug) run against 100ul of rabbit antiserum to UT5 with the incorporation of an intermediate gel containing a)no antiserum, b)antiserum (30ul) to A2. The gel was stained with Coomassie blue.

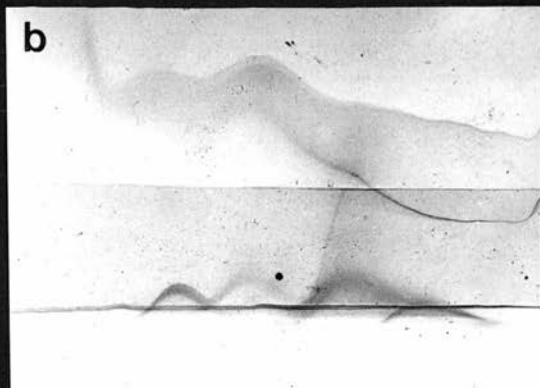


Figure 5.6

The agar gel diffusion patterns obtained with a) antiserum to All and b) antiserum to UT6 diffused against the PWE antigens of 1) UT5, 2) UT6, 3) UT7, 4) UT10, 5) the HE antigen to All and 6) the PWE antigen UT19.



## SECTION 5.2

### The serogrouping of IHA negative isolates of *P.haemolytica* by countercurrent immunoelectrophoresis.

The results obtained in the previous section showed that the serogroup antigens of IHA negative isolates were demonstrable by AGD and CIE. However, both of these tests are time consuming and other disadvantages, such as the amount of antisera required for CIE and the insensitivity of AGD, make them unsuitable as a routine laboratory test for serogrouping large numbers of isolates. Countercurrent immunoelectrophoresis (CCIE) utilises small volumes of reagents and as a result of electrophoresis is a rapid and sensitive test. The potential of this method was examined.

The CCIE method is described in Materials and Methods. The SSE and PWE antigens of the six isolates were run against antisera to UT6 and UT18. The patterns obtained again confirmed the presence of two groups with no heterologous reactions present. This demonstrated the suitability of the CCIE method for serogrouping the isolates. However, as both SSE and PWE are relatively complicated extracts to prepare, a better antigen would be preferable. As heating *P.haemolytica* cells at 56°C released IHA antigen into solution (personal observation), it was possible that heating IHA negative cells at 56°C for 45 min would have a similar effect. The supernate from this procedure was used as antigen (HE antigen). The method is fully detailed in Materials and Methods.

The HE antigens of the six isolates were examined by CCIE and

it was found that the reactions were similar to those obtained with SSE and PWE antigens. The antisera to the two representative isolates UT6 and UT18 were then used to examine all the other isolates in the collection. Three isolates, UT34, 43 and 44 reacted specifically with antiserum to UT6 and two isolates, UT8 and UT39, with UT18. No other HE antigens reacted. A further six isolates were selected from those which did not react and were used to hyperimmunise rabbits. By the continued use of this system of elimination and selection a total of ten groups have been identified in the collection of 31 isolates. The isolates present in each group and their origin are shown in Table 5.3.

An example of the precipitin line obtained with CCIE is shown (Fig. 5.7). The rabbit antiserum to isolate UT23 (groupIV) was absorbed with the cells of representative members of all other serogroups and reacted with the HE antigens of various isolates. It reacts specifically with UT23, UT30 and UT33. The precipitin reaction comprises two closely spaced precipitin lines.

The results of this experiment demonstrated that the 31 IHA negative isolates can be serogrouped by CCIE using HE antigens. There does not appear to be any correlation between the serogroup of the isolate and the species from which it was isolated.



Table 5.3

The distribution of IHA negative isolates into serogroups established by CCIE.

SEROGROUP	ISOLATE No.	ORIGIN <sup>1</sup>		TOTAL
		OVINE	BOVINE	
I	6,10,34,43,44	3	2	5
II	5,7,8,18,19,39	6	0	6
III	3,21,22,24,25	1	4	5
IV	23,30,33	2	1	3
V	2,42	1	1	2
VI	26,28	2	0	2
VII	29,31,36,37	4	0	4
VIII	27	1	0	1
IX	35,40	1	1	2
X	38	1	0	1

1 - Origin = Species from which isolate was recovered.

Figure 5.7

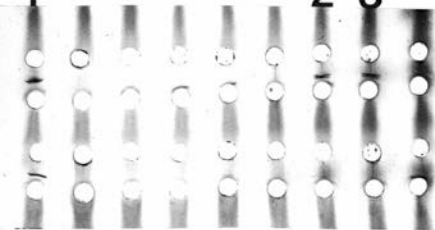
CCIE of antiserum to serogroup IV (a) reacted with the HE antigens (h) of several IHA negative isolates. The antiserum shows reactions with 1)UT23, 2)UT30 and 3)UT33.

1

2

3

h  
a



SECTION 5.3

The serogrouping of North American IHA negative isolates of *P.haemolytica* by CCIE and the correlation of these serogroups with those found by RPA.

The ten IHA negative isolates of *P.haemolytica* examined by Frank (1980) together with 3 rabbit RPA serotype antisera were obtained from Dr. G. Frank, (National Animal Disease Centre, Ames, Iowa, USA.). One isolate, 148-78, was lost in transit and unfortunately this was the sole representative of Frank's group 3. The surviving 9 isolates were reconstituted in nutrient broth, subcultured on 7% SBA and HE antigens prepared.

All the isolates obtained from Dr. Frank did not react with homologous antisera nor with IHA typing sera in the IHA test. The three RPA antisera did not react with the 15 prototype IHA serotype antigens.

When the HE antigens of Frank's isolates and the antisera to those isolates were tested in CCIE the results obtained were identical to those reported by Frank (1980) for the RPA reaction (Table 5.4).

At the time these examinations were carried out only antisera to the first 5 of MRI's IHA negative serogroups were available and none of these reacted with the HE antigens of Frank's isolates in CCIE. However, when the representative strains of the 10 groups were examined, it was found that UT35 (Group IX) reacted with antiserum to isolate 282-76 (Table 5.5).

The results indicated that there is complete correlation between CCIE and RPA methods in the serogrouping of Frank's

isolates. As MRI Group IX is identical to Frank's group 2 it means that Frank's other two groups can be added to the 10 from MRI and the number of serogroups extended to 12.

Table 5.4

Results of RPA and CCIE tests on Frank's IHA negative isolates when reacted with antisera to Frank's three "serotypes".

ISOLATE	RPA1 WITH ANTISERA TO			CCIE WITH ANTISERA TO		
	194.76	282.76	148.78	194.6	282.76	148.76
1. 171-85	+	-	-	+	-	-
2. 194-76	+	-	-	+	-	-
3. 253-76	+	-	-	+	-	-
4. 275-76	+	-	-	+	-	-
5. 288-76	+	-	-	+	-	-
6. 290-76	+	-	-	+	-	-
7. 282-76	-	+	-	-	+	-
8. 88-76	-	+	-	-	+	-
9. 148-78	-	+	-	-	+	-

1 - Frank's results (1980)

+ = Positive reaction

- = Negative reaction

Table 5.5

The reaction of Moredun IHA negative serogroup HE antigens with antisera to Frank's "serotypes" in CCIE.

ANTISERA TO	CCIE HE ANTIGENS OF										FRANK'S	
	MOREDUN										SEROTYPES	
	SEROGROUP											
	I	II	III	IV	V	VI	VII	IX	X		194-76	282-76
194 - 76	-	-	-	-	-	-	-	-	-		+	-
282 - 76	-	-	-	-	-	-	-	+	-		-	+
148 - 78	-	-	-	-	-	-	-	-	-		-	-

+ = Positive reaction

- = Negative reaction

#### SECTION 5.4

##### The protective immunity of SSE preparations from IHA negative isolates.

Immunity to some of the IHA positive serotypes of P.haemolytica has been shown to be serotype specific (Gilmour et al, 1979, 1983) and work described in this thesis confirms this for P.haemolytica A1 (section 1) and A6 (section 4). This immunity in mice is conferred by a serotype-specific antigen.

If the IHA negative isolates are important in pathogenesis it would be beneficial to be aware of the possible immunising properties of the serogroup antigens.

SSE was prepared from IHA negative isolates, UT5 and UT18 (group II) and UT6 (group I), made into vaccines with aluminium hydroxide and oil and tested in the mouse model for P.haemolytica infection as described in Materials and Methods.

In the first experiment mice vaccinated with UT5 SSE (284ug per dose) were challenged with UT5, UT7, UT18, UT19 (group II), UT6, UT10 (group I) and serotype A2. The results are shown in Table 5.6. These show that protection against all of group II isolates except for UT19 is significant ( $P=0.001$ ) and that significant protection extends to challenge with group I isolates ( $P=0.01$ ). There was no protection against A2 challenge.

In a second experiment mice vaccinated with UT6 SSE or UT18 SSE (56.8ug per dose of vaccine) were challenge with UT6, UT18 and A1. The results are shown in Table 5.7. Both antigen preparations gave protection against homologous challenge ( $P=0.01$  for UT6 and  $P=0.05$  for UT18). The SSE antigen of UT18



conferred protection against UT6 challenge ( $P=0.05$ ) but UT6 SSE did not confer protection against UT18 challenge. Neither antigen protected against A1 challenge.

The results of the first experiment indicate that although the serogroup antigens are involved in protection they are not the sole antigens. The cross-protection seen between the serogroups and the complete lack of protection to UT19 challenge suggest that other antigens play a role in protection. The second experiment, confined to two serogroups demonstrates again that cross-protection is evident between the groups. However, the cross-protection here is one way, group II antigens protect against group I strains but not the converse. There is some indication that UT6 is a weak pathogen in mice as the control mice challenged with this isolate had a mean liver count of only  $\log_{10}$  4.04 cfu in the first experiment and  $\log_{10}$  3.79 cfu in the second. These are the lowest counts in any of the challenges. The cross-protection found between serogroups did not extend to the IHA positive serotypes, A1 and A2.

Table 5.6

Counts<sup>1</sup> of P.haemolytica in the livers of unvaccinated control mice and mice vaccinated with P.haemolytica UT5 SSE 6h after challenge.

CHALLENGE ISOLATE	CHALLENGE DOSE (cfu per dose)	MEAN LIVER COUNT AFTER CHALLENGE IN		p <sup>2</sup>
		CONTROL GROUP	VACCINATED GROUP	
UT5 (Group II)	5.83	6.37±1.5	2.74±0.28	0.001
UT7 (Group II)	5.85	6.70±0.9	3.60±1.0	0.001
UT18(Group II)	6.1	6.80±0.4	4.60±1.6	0.001
UT19(Group II)	6.28	6.70±0.3	5.50±1.6	NS <sup>3</sup>
UT6 (Group I )	6.3	4.04±1.26	2.76±0.2	0.01
UT10(Group I )	6.6	7.90±0.5	6.50±1.7	0.01
A2	6.24	7.30±0.7	7.60±0.5	NS

1. Counts = cfu expressed as log<sub>10</sub> values

2. P = vaccinated group compared with control group in Mann-Whitney ranking test

3. NS = Not significant

Table 5.7

Counts<sup>1</sup> of P.haemolytica in the livers of unvaccinated control mice and mice vaccinated with UT6 or UT18 SSE after challenge with homologous and heterologous P.haemolytica isolates.

VACCINE (56.8ug)	CHALLENGE ISOLATE	CHALLENGE DOSE (cfu)	MEAN LIVER COUNT		IN	P	2
			CONTROL GROUP	VACCINATED GROUP			
UT6 SSE	UT6 (Group I )	6.18	3.79±1.1	2.73±0.09	0.01		
UT6 SSE	UT18(Group II)	5.88	4.70±1.6	4.70±1.36	NS		
UT6 SSE	Al	6.08	6.25±1.7	7.38±0.50	NS		
UT18 SSE	UT6 (Group I )	6.18	3.79±1.1	3.03±0.40	0.05		
UT18 SSE	UT18(Group II)	5.88	4.70±1.6	2.80±0.9	0.05		
UT18 SSE	Al	6.08	6.25±1.7	6.17±2.1	NS		

1. Counts = cfu expressed as  $\log_{10}$  values

2. P = Vaccinated group compared with control group in Mann Whitney ranking test

### SECTION 5.5

#### The pathogenicity of IHA negative isolates for mice and SPF lambs.

Although Biberstein et al (1960) and Aarslef et al (1970) described agglutination studies with IHA negative isolates they did not study the pathogenicity of the isolates.

As IHA negative isolates can be subdivided on the basis of serogroup antigens it is possible that they also vary in their pathogenicity. Their relative pathogenicity for mice can be established by the mouse LD<sub>50</sub> test. However, as the mouse is not the natural host a much more relevant assessment can be carried out in sheep by the SPF lamb model for infection developed at MRI by Sharp et al (1978).

The mouse LD<sub>50</sub> of a representative strain from each of nine serogroups, I to IX, was measured using the method given in Materials and Methods. The method of infection used in SPF lambs has also been described in Methods and Materials.

The mouse LD<sub>50</sub> results are given in Table 5.8. There are marked differences in the virulence of the isolates. Isolate UT2 is the most virulent (LD<sub>50</sub>= $2.58 \times 10^5$  cfu) with UT3 and UT5 slightly less virulent (LD<sub>50</sub>= $1.08 \times 10^6$  cfu and  $4.2 \times 10^6$  cfu respectively). All the rest have LD<sub>50</sub> values of  $> 1 \times 10^7$  cfu.

On the basis of these results, which were the average of two assessments, an isolate of high virulence, UT2, and an isolate of lower virulence, UT18, were chosen to challenge SPF lambs.

After challenge of two groups of 4 SPF lambs no clinical, pathological or bacteriological evidence of pasteurellosis was found in the lungs. P.haemolytica isolates recovered from the

upper respiratory tract of lambs in both groups were tested in CCIE and found to correspond to the serogroup of the strains administered in the aerosols.

Representative members of nine IHA negative serogroups have been shown to differ in their virulence for mice. However, even the most virulent of these, UT2, does not appear to be pathogenic in the SPF lamb model for P.haemolytica infection.

Table 5.8

Mouse LD<sub>50</sub> values for representative isolates of IHA negative serogroups I to IX.

SEROGROUP	ISOLATE	LD <sub>50</sub> <sup>1</sup>
I	UT6	6.21 X 10 <sup>7</sup> cfu
II	UT18	1.1 X 10 <sup>7</sup> cfu
III	UT3	1.7 X 10 <sup>6</sup> cfu
IV	UT30	3.1 X 10 <sup>7</sup> cfu
V	UT2	2.58 X 10 <sup>5</sup> cfu
VI	UT26	6.48 X 10 <sup>7</sup> cfu
VII	UT29	2.2 X 10 <sup>7</sup> cfu
VIII	UT27	6.2 X 10 <sup>7</sup> cfu
IX	UT35	2.2 X 10 <sup>6</sup> cfu

1. LD<sub>50</sub> = Mean of two measurements.

## DISCUSSION

The first experiments showed that all of the IHA negative strains belonged to the A biotype and that the 31 strains could be separated into ten serogroups. These results are in broad agreement with the findings of Frank (1980). He found that three distinct serotypes could be identified with the RPA test among ten isolates from the nasal cavities of cattle. Although the initial RPA results of this study were promising, in that two groups were discovered in six isolates, the test was not satisfactory when applied to all the isolates in the collection. This may have been due to cross-reacting antigens on the surface of the IHA negative strains which have been described by Biberstein et al (1960) and Aarslef et al (1970). Although cross-absorption was carried out with the two initial groups it may not have been effective in stopping agglutinating activity with other isolates.

The dominance of the IHA serotype antigen in CIE has already been demonstrated for IHA positive serotypes (Section 1.1 and 4.1) and a similar situation exists for IHA negative serogroup antigens. Two specific antigens or at least antigens that do not cross-react with heterologous antisera, are seen in CIE for serogroups I and II. Although CIE was not carried out on isolates from other groups the presence of distinct specific precipitin lines, in homologous reactions in CCIE, would seem to indicate that this observation with groups I and II holds for all the serogroups. The presence of these antigens in PWE suggests that they are similar to the serotype antigens of A1, A2 and A6 (Section 1 and 4) and that they are probably the same

type of molecule (i.e. a cell surface carbohydrate). However, as the IHA negative strain antigens do not react in the IHA test there must be important differences between these and the IHA positive antigens. The cross-reaction of antiserum between All and group I antigen is unusual as the HE antigen of All does not seem to contain the cross-reactive antigen.

The general lack of cross-reactivity that the serogroup antigens exhibit with IHA positive serotypes, with the exception of All, strongly suggests that IHA negative isolates are in groups distinct from the IHA positive serotypes. It has previously been suggested that IHA-negative isolates were in fact IHA positive serotypes which somehow lacked the soluble serotype antigen (Biberstein et al, 1960). Although this suggestion may explain the All-group I cross-reaction it does not seem to hold for the other serogroups.

The demonstration that CCIE separated Frank's 9 strains into the same serogroups that he found with RPA confirms his results and shows the usefulness of CCIE. The placing of Frank's isolate 282/76 in serogroup IX shows that some IHA negative serogroups are found in both North America and in Britain.

As 12 serogroups (10 at MRI and 2 in the USA) have now been identified in only 41 isolates it would appear that there may be as many if not more IHA negative serogroups as IHA positive serotypes. This is interesting as IHA negative isolates only account for 11% of all isolates referred for serotyping (Fraser et al, 1982a).

The recovery of IHA-negative isolates from pneumonic lesions



suggests that they are pathogenic for sheep but no experimental evidence has yet been produced to substantiate this theory. Virulence studies in mice indicated that isolate UT2 was highly virulent compared to the rest although the LD<sub>50</sub> ( $2.58 \times 10^5$  cfu) was similar to that recorded for IHA positive serotype A1 ( $2 \times 10^5$  cfu) by Evans (1979). However, it was not pathogenic for SPF lambs which have been used successfully for demonstrating the pathogenicity of A biotype IHA positive serotypes A1, A2, A6, A9 (Gilmour et al, 1979; Gilmour et al, 1983). This suggests that IHA negative isolates may have reduced pathogenicity compared to the IHA positive isolates although other IHA negative isolates should be tested before coming to firm conclusion. Gilmour (personal communication) has found that a T biotype, (serotype T10) isolate was not pathogenic in this SPF lamb model and it may be that although IHA negative and T biotype isolates are recovered from pneumonic lesions they are not the primary P.haemolytica organism and are present in conjunction with a more pathogenic A serotype.

The immunisation of mice with SSE from group I and group II isolates showed that although protection was associated with the serogroup antigen where UT6 SSE (group I) was used there was some indication with UT5 and UT18 (group II) SSE's that cross-protection against group I was present. The cross-protection was probably due to factors other than the serogroup antigen. The whole question of cross-protecting antigens relating to IHA positive and IHA negative isolates is discussed in Section 4. However another factor in apparent cross-protecting may be the weak pathogenicity of the UT6

isolate which has been discussed earlier (Section 5.4).

This aspect of mouse pathogenicity and its relationship to possible pathogenicity in the sheep is one which raises questions about the validity of the mouse model for P.haemolytica studies. The converse situation existed for serotype A2 where it had been impossible to demonstrate protection in mice but protection with SSE and HKO has been shown in sheep (Glimour et al, 1983). It may be that, although the mouse model can be usefully employed to screen for possible protective

antigens, it cannot be assumed that the same response to these antigens will occur in sheep and vice versa. There is an obvious need to establish a correlation between mouse and sheep results to avert any possible misinterpretation. The mouse ELISA described in section 3 may offer one avenue of future interest as the titre of antibodies to specific antigens may correlate with a degree of protection as has been already shown for Neisseria meningitidis vaccines. Goldschneider et al (1969) have demonstrated that the level circulating antibodies in humans is proportional to the bactericidal effect of the sera and hence its protective ability.

### GENERAL DISCUSSION

The primary aims of this study were to identify, isolate and characterise the protective antigen or antigens of P.haemolytica A1 and, if found, to ascertain whether antigens with similar properties existed within the other A biotype serotypes. In the course of this study the discovery of serotypes within the untypable group of P.haemolytica isolates from cattle in the USA was reported by Frank (1980). The techniques used in examination of A1 antigens were applied to those of the untypable isolates recovered from both sheep and cattle in the UK.

The general discussion collates information obtained during the course of this study and suggestions are made for future research which may provide additional useful data.

The importance of a phenol-water extracted carbohydrate antigen from A1 as a protective antigen was demonstrated in mice and furthermore the same type of extract made from A6 cells contained the protective antigen for this serotype. This is in agreement with earlier work reported by Cameron (1966) who showed that LPS antigen, obtained by phenol-water extraction of whole cells, conferred protection. However, it is in disagreement with the findings of Tadayon and Lauerman (1981) who did not obtain protection in mice or hamsters with LPS but found that a potassium thiocyanate (KSCN) extract was the most effective. These workers did not give details of the serotype or the challenge method used so it is difficult to assess the results in relation to those presented here.

One of the aims of this thesis was to characterise the

protective antigen and although the LPS has been identified and shown to contain the serotype specific antigen it has not been possible to relate the composition of the molecule to the differences that are detected serologically between the serotypes. Structural analyses using methylation of molecules should be carried out to determine the basis for the serotype differences. Methylation analysis has already been used to determine the LPS structure of Yersinia pseudotuberculosis type III (Gorshkova et al, 1980).

However, the CIE results indicated that more than one antigen was present in the PWE of A1 and it would be essential to isolate the serotype antigen in a pure form before proceeding. Although it was not possible to do this by affinity chromatography other methods of separation, such as ion exchange columns or isoelectric focussing, might be successful. The full identification of the two heptoses present in the PWE of the three serotypes was not achieved in this work although alditol acetate derivatives from a number of different heptoses were compared on GC with them. Further work on this may reveal whether these heptoses are peculiar to P.haemolytica, and whether they form part of the same molecule or number of different molecules on the surface of P.haemolytica.

Previous studies on P.haemolytica LPS have concentrated on

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the endotoxic aspects of this component. Keiss et al (1964) working on a bovine isolate calculated that 25% of the total cell dry weight was endotoxin. This was based on measurement of

the dermatotoxic effect in rabbits and the haemodynamic effect in sheep and would appear to be exaggerated.

A more recent report by Rimsay et al (1981) on serotype A7 showed that LPS extracted from P.haemolytica by the phenol/chloroform/ether (PCE) method of Galanos et al (1969) for rough LPS or the hot phenol-water method of Westphal et al (1952) for smooth LPS accounted for 10% of the dry cell weight and had different biological effects. The isolated polysaccharide from the rough LPS was less endotoxic than smooth LPS but was still able to induce non specific protection in mice against challenge exposure with S.typhimurium.

The authors suggested that the polysaccharide from rough LPS could possibly be used to stimulate non specific protection in animals. However, no measurement of specific P.haemolytica antigenicity was attempted and it is possible that the polysaccharide of the rough LPS does share some antigenic determinants with S.typhimurium or that the PCE treatment has exposed core determinants which would otherwise have been hidden in the smooth LPS.

The results of the mouse protection tests indicated that on some occasions the protection afforded by the PWE was not as strong as that given by SSE (Sections 1.2, 4.4). It is known that some purified bacterial polysaccharides are notoriously poor immunogens (Schneerson et al, 1980a) and this may explain the apparent weaker immunogenicity of the PWE.

The use of an adjuvant may improve the immunogenicity and Wells et al (1979) have shown that the serum antibody response

to P.haemolytica Al SSE antigens is higher when the antigens are first absorbed onto alhydrogel then emulsified in incomplete Freund's adjuvant than when the antigens are emulsified in complete Freund's adjuvant, incomplete adjuvant or absorbed onto alhydrogel alone.

However a more interesting approach is that taken by Svenson et al (1979) who prepared artificial S.typhimurium vaccines by covalently linking "O" antigen polysaccharides to diphtheria toxin or isolated porin proteins from S.typhimurium. These were compared for protective efficacy with a third vaccine containing only porins, against homologous S.typhimurium challenge in mice. The vaccine containing "O" antigen linked to porins was the most effective but porins alone were also protective. However, porins are unlikely candidates for vaccine components as they are difficult to prepare.

Experiments with the "O" antigen linked to a much more readily accessible protein, bovine serum albumin, were performed and also found to be successful (Jorbeck et al, 1981). Schneerson et al (1980b) has reported that H.influenzae type b polysaccharide antigen is a more effective immunogen when linked to bovine serum albumin and suggests that this may be due to a change from T independent to a T dependent antigen.

The protective property of the PWE antigen suggests that it should be considered as a possible vaccine component for sheep vaccines against pasteurellosis although the preparation procedure might be too elaborate for commercial firms. However, the trend displayed by companies involved in the production of vaccines for animal health is towards multivalent vaccines

comprising a number of different organisms. One of these contains antigens giving immunity to seven different clostridial related diseases and also the antigens from 8 serotypes of P.haemolytica (Heptavac P, Hoechst U.K., Walton, Milton Keynes, Bucks). If this trend is continued then the requirement will be for the refined antigens important in protection with all other superfluous material removed. Under these circumstances the PWE antigen may appear a more acceptable antigen and suggests that its effectiveness in sheep should be examined .

The presence of other protective antigens cannot be ignored and Cameron (1966) indicated that the protein as well as the carbohydrate antigens were important in immunity. Biberstein and Thompson (1965) concluded that other antigens apart from the IHA antigen played a role in immunogenicity to A2. It may be that the situation is similar to that found with N.meningitidis where 3 main serogroups can be identified on the basis of their capsular antigen. The capsular antigens of two of the groups, A and C, are protective immunogens when challenged by their respective serogroups but the B antigen does not confer immunity. It has been shown that immunity to this group resides in an outer membrane protein (Goldschneider, et al 1969). The protein antigens of all the P.haemolytica serotypes should be examined for possible protective immunogenicity.

The use of the Western blotting method (Towbin et al, 1979) in the examination of bacterial antigens has been reported (Virji et al, 1983) and the SDS-PAGE separation combined with serological examination makes this a powerful tool in the analysis of antigens. A detailed antigenic study of the



antigens on the cell surface of P.haemolytica could be undertaken utilising a range of antisera from sheep which have differing immune status. This could yield information on the antigens which are important during infection and hopefully in immunity.

Another approach would be to produce monoclonal antibodies (Kohler and Milstein, 1975) to P.haemolytica antigens and to use these as probes for detecting and analysing the surface antigens. Apicella et al (1981) have applied the monoclonal antibody technique to the analysis of N.gonorrhoeae and N.meningitidis LPS antigens and shown the presence of a shared determinant on these molecules. Monoclonal antibodies raised against P.haemolytica antigens could be utilised in a number of ways such as the identification of antigens when combined with the Western blotting technique (Virji et al, 1983), location of antigens on the cell surface on electron microscopy, incorporation onto an immunosorbent column for the removal of the antigen from a complex mixture of antigens (Momoi et al, 1980) and perhaps even the passive protection of mice to determine any protection endowed by the antibody.

Protection against challenge by P.haemolytica A2 was not demonstrated consistently in mice given inactivated vaccine but with mice surviving an LD<sub>50</sub> dose of A2 there appeared to be solid immunity. This suggests that protection requires antigens which are either particularly labile or not extractable and that they can only be presented properly on live cells. If either is true then the protection might best be effected by vaccination with live bacteria. Wei and Carter (1978) have shown that



streptomycin dependent mutants of P.multocida can be produced and that they can stimulate immunity in mice and rabbits when given as a vaccine. More recently Kucera et al (1983) have reported that a strain of P.haemolytica A1, which had been grown in media containing acriflavine was attenuated as measured by a decrease in virulence for mice. This strain was used to immunise cattle by intra muscular inoculation and shown to protect the cattle against intratracheal challenge. It may be possible to treat A2 strains in either of the two ways described to produce vaccine strains.

Another possibility is that the cytotoxin associated with P.haemolytica, which has been described in cattle strains by Maheswaren et al (1980), is an important pathogenic effector mechanism and immunity to this would be important in protection. The cytotoxin is specifically active against the phagocytic cells of ruminants but not for other cells in the ruminant or the cells of other species which have been tested. (Sutherland et al, 1983). Interestingly ultraviolet killed cells, LPS and SSE of A1 do not exhibit cytotoxic activity so it is not thought that the toxin is a cell surface component but that it has to be elaborated by living cells. This cytotoxin is only present in the supernatant fluid of growing cultures or detected when living cells are present. A. Sutherland (personal communication) has demonstrated that the toxin can be neutralised by convalescent sheep sera and by sera raised against live cells and must therefore be produced in vivo. Immunization against this toxin may offer the host a defence against one of the bacterium's earliest effects, that of killing

the phagocytic macrophages in the lung. Again this would be produced by a live vaccine strain. The apparent specificity of the toxin for the phagocytic cells of ruminants may be connected with limited range of species in which P.haemolytica is naturally pathogenic.

The ELISA methods which were developed in this work could be further improved if the serotype antigen found in the PWE was purified as suggested above. The absorption studies (Section 3.1) showed that a large proportion of the serotype specific activity resided in the PWE antigens. However, the observation that SPF lambs, even before vaccination, had antibody that reacted with A9 PWE suggests that the sensitivity of the test may detect the presence of antigens in P.haemolytica which are present in other bacterial species. Although it may be due to an individual antigen it could be the result of shared determinants present on the LPS, as is the case between Vibrio cholerae and E.coli, Salmonella and Citrobacter species (Winkle et al, 1972).

It would be difficult to remove the cross-reacting determinant in these cases.

The serotype specific ELISA could be applied to the measurement of antibodies against a single serotype in serum from conventional animals vaccinated with a multivalent pasteurella vaccine or in the sera of sheep undergoing infection with a P.haemolytica strain. Although this is also possible with the IHA the ELISA has the combined attributes of its sensitivity and the capability of measuring different classes of antibody. This is of particular importance in the lung where immunity to P.haemolytica is thought to be important. Smith et al (1975)

showed that IgA was the major immunoglobulin in the ovine lung and it would seem important to establish the class and role of pulmonary antibodies to P.haemolytica in the immune animal as this would allow the development of vaccines designed to specifically stimulate the correct type of response.

The ELISA technique using very small volumes (10ul) of mouse blood was highly sensitive for measuring antibody titres to P.haemolytica in mouse sera. This could extend the usefulness of the mouse model for P.haemolytica infection (Evans and Wells, 1979a) by affording a screening procedure for potential vaccine components such as the A1 PWE which was shown to stimulate antibodies against itself (Section 3). With this ELISA it would also be possible to measure the minimum dose of antigen required to stimulate an antibody response and to correlate antibody titre with a protective dose. This in turn may lead to the same procedure in sheep where evaluation of the protective capability of a vaccine could be carried out by measurement of an antibody response and not by the challenge of the animal. This has been used for the determination of immunity against N.meningitidis (Goldschneider et al, 1969) where bactericidal antibodies are measured.

The examination of IHA negative isolates was stimulated by the large increase in the prevalence of these strains between 1978 and 1982 from 5% to 11% (Thompson et al, 1977; Fraser et al, 1983) and by a report (Frank, 1980) that distinct serotypes had been identified among a group of IHA negative strains recovered from cattle. The demonstration of 10 serogroups within 31 strains from the MRI collection using CCIE indicates the

potentially large number of serogroups which may exist. Although an immunological study was carried out on the serogroup specific antigens the chemical analysis of these antigens remains to be done. This may give some information on an important difference between these strains and IHA positive strains; for example why they do not react in the IHA test. The binding of the IHA antigen is a reversible phenomenon (personal observation) indicating a specific receptor or binding site on the RBC membrane. The serogroup antigen of the IHA-negative strains may lack a reacting group for the receptor or binding site or be a completely different antigen from the IHA positive serotype antigen.

The results obtained in the examination of pathogenicity of these strains do not clarify their possible role in the aetiology of pasteurellosis. As only two strains were tested there is insufficient evidence to state that all IHA negative strains are not pathogenic but other IHA negative strains should be examined in the experimental SPF lamb model.

CCIE is a convenient method for serotyping IHA negative strains and if the strains can be monitored, using this method, in the field then a proper assessment of the role of these strains in disease can be made.

The serogroups were numbered with Roman numerals to differentiate them from IHA positive strains. This should help avoid confusion if more IHA positive serotypes are discovered as these can be given numbers consecutive to those existing already and be easily identified as IHA positive serotypes. The standardisation of the nomenclature is important and to this end

it may be sensible to have one or two laboratories which will perform the serology and designate new serotypes or serogroups as they come to light.

To conclude, the major points arising from this work are: mice can be protected against challenge by P.haemolytica A1 and A6 with a carbohydrate antigen which is probably LPS; this antigen can be used in a serotype specific micro ELISA; mice cannot be consistently protected against P.haemolytica A2 challenge with extracts of the cells surface of the bacterium, but solid protection was shown after challenge with an LD<sub>50</sub> of A2; and IHA negative strains of P.haemolytica can be serogrouped using CCIE but their pathogenicity for sheep needs to be further assessed.

## REFERENCES

- AARSLEFF, B., BIBERSTEIN, E.L., SHREEVE B.J. & THOMPSON, D.A. (1970).  
A study of untypable strains of Pasteurella haemolytica.  
Journal of Comparative Pathology. 80, 493-498.
- AHLSTEDT, S & LINDHOLM, P. (1977).  
Antibody response to Escherichia coli O antigens and influence of the protein moiety of the endotoxin.  
Immunology. 33, (5), 629-633.
- AL-SULTAN, I.A. (1982)  
Pasteurella haemolytica in sheep: Some studies on pathogenesis  
PhD. Thesis, University of Edinburgh.
- AMES, G.F.L. (1974).  
Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs.  
Journal of Biological Chemistry. 249, 634-644.
- AMIES, C.R. (1951).  
The envelope substances of Pasteurella pestis.  
British Journal of Experimental Pathology. 32, 254-272.
- APICELLA, M.A., BENNET, K.M., HEMERATH, C.A & ROBERTS, D.E. (1981).  
Monoclonal antibody analysis of lipopolysaccharide from Neisseria gonorrhoeae and Neisseria meningitidis.  
Infection and Immunity. 34, 751-756.
- BARRETT, D.J., AMMAN, A.J., STENMARK, S. & WARA, D.W. (1980).  
Immunoglobulin G and M antibodies to pneumococcal polysaccharides detected by enzyme-linked immunosorbent assay.  
Infection and Immunity. 27, 411-417.
- BHASIN, J.L. & LAPOINTE-SHAW, L. (1980).  
Antigenic analysis of Pasteurella multocida (serotype 1) by crossed immunoelectrophoresis: characterisation of cytoplasmic and cell envelope associated antigens.  
Canadian Journal of Microbiology 26, 676-689.
- BIBERSTEIN, E.L. (1978).  
Biotyping and serotyping of Pasteurella haemolytica.  
Methods in Microbiology. 10, 253-269.
- BIBERSTEIN, E.L. & FRANCIS, C.K. (1968).  
Nucleic acid homologies between the A and T types of Pasteurella haemolytica.  
Journal of Medical Microbiology. 1, 105-108.
- BIBERSTEIN, E.L. & GILLS, M.G. (1962).  
The relation of antigenic types to the A and T types of Pasteurella haemolytica.  
Journal of Comparative Pathology. 72, 316-320.



BIBERSTEIN, E.L., GILLS, M.G. & KNIGHT, H. (1960).  
Serological types of Pasteurella haemolytica.  
Cornell Veterinarian. 50, 283-300.

BIBERSTEIN, E.L. & KENNEDY, P.C. (1959).  
Septicaemic pasteurellosis in lambs.  
American Journal of Veterinary Research. 20, 94-101.

BIBERSTEIN, E.L. & KIRKHAM, C. (1979).  
Antimicrobial susceptibility patterns of the A and T types of  
Pasteurella haemolytica.  
Research in Veterinary Science. 26, 324-328.

BIBERSTEIN, E.E., NISBET, D.I. & THOMPSON, D.A. (1967).  
Experimental pneumonia in sheep.  
Journal of Comparative Pathology. 77, 181-192.

BIBERSTEIN, E.L. & THOMPSON, D.A. (1965).  
Type specificity of immunity to Pasteurella haemolytica  
infection in mice.  
Journal of Comparative Pathology. 75, 331-337.

BIBERSTEIN, E.L. & THOMPSON, D.A. (1966).  
Epidemiological studies on Pasteurella haemolytica in sheep.  
Journal of Comparative Pathology. 76, 83-94.

BOIVIN, A. & MESROBEANU, I. (1933).  
Contribution a l'etude de la composition clinique des bacteries.  
Substances azotees et phosphorees "acido-solubles".  
Comptes Rendus des Seances de la Societe de Biologie et des  
Filiales. 114, 302-307.

BOOTH, B.R. & CURTIS, N.A.C. (1977).  
Separation of the cytoplasmic and outer membrane of Pseudomonas  
aeruginosa.  
Biochemical, Biophysical Research Communications. 74,  
1168-1176.

BRADLEY, S.G. (1979).  
Cellular and molecular mechanisms of action of bacterial  
endotoxins.  
Annual Review of Microbiology. 33, 67-94.

BRAGG, P.D. & HOU, C. (1972).  
Organisation of proteins in the native and reformed outer  
membrane of Escherichia coli.  
Biochemica et Biophysica Acta. 274, 478-488.

BRAUN, V. & REHN, K. (1969).  
Chemical characterization, spatial distribution and function of  
a lipoprotein (murein-lipoprotein) of the E.coli cell wall. The  
specific effect of trypsin on the membrane structure.  
European Journal of Biochemistry. 10, 426-438.

BRUBAKER, R.R., HELLERQUIST, C.G. LINDBERG, B. & SAMUELSON, K.  
(1973).  
Studies on lipopolysaccharides from Yersinia pseudotuberculosis.  
Contributions to Immunology and Microbiology. 12, 6-9.

- BURRELLS, C., WELLS, P.W. & DAWSON, A.McL. (1979).  
The quantitative estimation of antibody to Pasteurella haemolytica in sheep sera using a micro enzyme-linked immunosorbent assay (ELISA).  
Veterinary Microbiology. 3, 291-301.
- CALVER, G.A., KENNY, C.P. & LAVERGNE, G. (1976).  
Iron as a replacement for mucin in the establishment of meningococcal infection in mice.  
Canadian Journal of Microbiology. 22, 1832-1838.
- CAMERON, C.M. (1966).  
The haemagglutination test and immunity to Pasteurella haemolytica.  
Journal of the South African Veterinary Medical Association. 37, 165-170.
- CAMERON, C.M. & SMIT, G. (1970).  
Immune response of rabbits, mice and sheep to polyvalent Pasteurella vaccine.  
Onderstepoort, Journal of Veterinary Research. 37, 217-224.
- CARLSSON, H.E., HURVELL, B. & LINDBERG, A.A (1976).  
Enzyme-linked immunosorbent assay (ELISA) for titration of antibodies against Brucella abortus and Yersinia enterocolitica.  
Acta Pathologica et Microbiologica Scandinavica, C. 84, 168-176.
- CARTER, G.R. (1967).  
Pasteurellosis: Pasteurella multocida and Pasteurella haemolytica.  
Advances in Veterinary Science. 11, 321-379.
- CASSIDY, J.T., JOURDIAN, G.W. & ROSEMAN, S. (1966).  
Sialidase from Clostridium perfringens.  
In S.P. Colowick and N.O. Kaplan (ed), "Methods in Enzymology"  
Vol 8. Academic Press, New York and London.
- CHEN, P.S., TORIBARA, T.Y. & WARNER, H. (1956).  
Micro-determination of phosphorus.  
Analytical Chemistry. 28, 1756-1758.
- COLLINS, F.M. & CAMPBELL, S.G. (1982).  
Immunity to intracellular bacteria.  
Veterinary Immunology and Pathology. 3, 5-66.
- COSTERTON, J.W. (1974).  
Structure and function of the cell wall envelope of Gram-negative bacteria.  
Bacteriological Reviews. 38, 87-110.
- COSTERTON, J.W. (1979).  
The role of electron microscopy in the elucidation of bacterial structure and function.  
Annual Review of Microbiology. 33, 459-479.



- COSTERTON, J.W., IRVIN, R.T. & CHENG, K.J. (1981).  
The role of bacterial surface structures in pathogenesis.  
C.R.C. Critical Reviews in Microbiology. September 1981.  
303-338.
- CROSSON, F.J., WINKLESTEIN, J.A. & MOXON, R.E. (1978).  
Enzyme-linked immunosorbent assay for detection and quantitation  
of a capsular antigen of Haemophilus influenzae type b.  
Infection and Immunity. 22, 617-619.
- DAHLBERG, T. & BRANEFORS, P. (1980).  
Enzyme-linked immunosorbent assay for titration of Haemophilus  
influenzae capsular and "O" antigen antibodies.  
Journal of Clinical Microbiology. 12, (2), 185-192.
- DAVIES, D.H., HERLEG, M., JONES, B.A.H. & THURLEY, D.C. (1981).  
The pathogenesis of sequential infection with para influenza  
virus type 3 and Pasteurella haemolytica in sheep.  
Veterinary Microbiology. 6, 173-182.
- DENNIS, S.M. (1974).  
Perinatal lambs mortality in Western Australia. 4 - Neonatal  
infections.  
Australian Veterinary Journal . 50, 511-514.
- DE PAMPHILIS, M.L. & ADLER, J. (1971).  
Attachment of flagellar basal bodies to the cell envelope:  
specific attachment to the outer, lipopolysaccharide membrane  
and the cytoplasmic membrane.  
Journal of Bacteriology. 105, 396-407.
- DIENA, B.B., LIOR, H., RYAN, A., KROL, P., JOHNSON, E.M. &  
BARON, L.S. (1979).  
Mouse protective capabilities of Escherichia coli hybrids  
expressing Salmonella typhi antigens.  
Infection and Immunity. 24, (1), 90-93.
- DI RIENZO, J.M., NAKAMURA, K. & INOUE, M. (1978).  
The outer membrane proteins of Gram-negative bacteria:  
biosynthesis, assembly and functions.  
Annual Review of Biochemistry. 47, 481-532.
- DISCHE, J. (1947).  
A new specific color reaction of hexuronic acids.  
Journal of Biological Chemistry. 167, 189-198.
- DUBOIS, M., GILLIES, K.A., HAMILTON, J.K., REBERS, P.A. & SMITH,  
F. (1956).  
Colorimetric method for determination of sugars and related  
substances.  
Analytical Chemistry. 28, 350-356.
- DUGUID, J.P. (1951).  
The demonstration of bacterial capsules and slime.  
Journal of Pathology and Bacteriology. 63, 637-685.

DUNCOMBE, W.G. (1963).

The colorimetric micro-determination of long chain fatty acids.  
Biochemical Journal. 88, 7-10.

DUNGAL, N. (1931).

Contagious pneumonia in sheep.

Journal of Comparative Pathology. 44, 126-143.

DUNGWORTH, D.L. & CORDY, D.R. (1962).

The pathogenesis of ovine pneumonia.

1. The isolation of a virus of the PLV group.

Journal of Comparative Pathology. 72, 49-70.

DYSON, D.A., GILMOUR, N.J.L. & ANGUS, K.W. (1981).

Ovine systemic pasteurellosis caused by Pasteurella haemolytica biotype T.

Journal of Medical Microbiology. 14, 89-95.

ENGVAL, E. & PERLMANN, P. (1972).

Enzyme linked immunosorbent assay ELISA.

Journal of Immunology. 109, 129-135.

EVANS, H.B. (1979).

A study of the mechanisms of immunity to Pasteurella haemolytica infection.

PhD Thesis, University of Edinburgh.

EVANS, H.B. & WELLS, P.W. (1979a).

A mouse model of Pasteurella haemolytica infection and its use in assessment of the efficacy of P. haemolytica vaccines.

Research in Veterinary Science. 27, 213-217.

EVANS, H.B. & WELLS, P.W. (1979b).

Non specific resistance of mice to Pasteurella haemolytica.

Research in Veterinary Science. 27, 297-301.

FENSOM, A.H. & MEADOW, P.M. (1970).

Evidence for two regions in the polysaccharide moiety of the lipopolysaccharide of Pseudomonas aeruginosa 8602.

FEBS Letters. 9, 81-84.

FRANK, G.H. (1980).

Serological groups among untypable bovine isolates of Pasteurella haemolytica.

Journal of Clinical Microbiology. 12, 579-582.

FRANK, G.H. & WESSMAN, G.E. (1978).

Rapid plate agglutination procedure for serotyping Pasteurella haemolytica.

Journal of Clinical Microbiology. 7, 142-145.

FRASER, J., GILMOUR, N.J.L., LAIRD, S & DONACHIE, W. (1982a).

Prevalence of Pasteurella haemolytica serotypes isolated from ovine pasteurellosis in Britain.

Veterinary Record. 110, 560-561.

- FRASER, J., LAIRD, S. & GILMOUR, N.J.L. (1982b).  
A new serotype (biotype T) of Pasteurella haemolytica.  
Research in Veterinary Science. 32, 127-128.
- FINNE, J., LEINONEN, M. & MAKELA, P.H. (1983).  
Antigenic similarities between brain components and bacteria causing meningitis. Implications for vaccine development and pathogenesis.  
Lancet. 11, 355-357.
- FORSBERG, C.W., COSTERTON, J.W. & McLEOD, R.A. (1970).  
Separation and localization of cell wall layers of a Gram-negative bacterium.  
Journal of Bacteriology. 104, 1338-1353.
- FUKUSHI, K., ANACKER, R.L., HASKINS, W.T., LANDY, M., MILNER, K.C. & RIBI, E. (1964).  
Extraction and purification of endotoxin from enterobacteriaceae: A comparison of selected methods and sources.  
Journal of Bacteriology. 87, 391-400.
- GALANOS, C. & LUDENTZ, O. (1975).  
Electrodialysis of lipopolysaccharides and their conversion to uniform salt forms.  
European Journal of Biochemistry. 54, 603-610.
- GALANOS, C., LUDERITZ, O. & WESTPHAL, O. (1969).  
A new method for the extraction of R lipopolysaccharides.  
European Journal of Biochemistry. 9, 245-249.
- GENTRY, M.J., CORSTVET, R.E. & PANCIERA, R.J. (1982).  
Extraction of capsular material from Pasteurella haemolytica.  
American Journal of Veterinary Research. 43, 2070-2073.
- GHUYSEN, J.M. (1968).  
Use of bacteriolytic enzymes in determination of wall structures and their role in cell metabolism.  
Bacteriology Reviews. 32, 425-464.
- GILMOUR, J.S., JONES, G.E., KEIR, W.A. & RUE, A.G. (1982).  
Long term pathological and microbial progress in sheep of experimental disease resembling atypical pneumonia.  
Journal of Comparative Pathology. 92, 229-238.
- GILMOUR, N.J.L. (1978a).  
Pasteurellosis in sheep.  
Veterinary Record. 102, 100-102.
- GILMOUR, N.J.L. (1978b).  
The role of pasteurellae in respiratory disease of cattle, p356-362. In W.B. Martin (ed), Respiratory Diseases of Cattle. Martinus Nijhoff, The Hague.
- GILMOUR, N.J.L. (1980).  
Pasteurella haemolytica infections in sheep.  
The Veterinary Quarterly. 2, (4), 191-198.

- GILMOUR, N.J.L & BROTHERSTON, J.G. (1963).  
Clinical and pathological findings in an outbreak of pneumonia in sheep.  
Journal of Comparative Pathology. 73, 329-335.
- GILMOUR, N.J.L., MARTIN, W.B., SHARP, J.M., THOMPSON, D.A. & WELLS, P.W. (1979).  
The development of vaccines against pneumonic pasteurellosis in sheep.  
Veterinary Record. 104, 15.
- GILMOUR, N.J.L., MARTIN, W.B., SHARP, J.M., THOMPSON, D.A., WELLS, P.W. & DONACHIE, W. (1983).  
Experimental immunisation of lambs against pneumonic pasteurellosis.  
Research in Veterinary Science. 35, 80-86.
- GILMOUR, N.J.L., SHARP, J.M. & GILMOUR, J.S. (1982).  
Effect of oxytetracycline therapy on experimentally induced pneumonic pasteurellosis in lambs.  
Veterinary Record. 111, 97-99.
- GILMOUR, N.J.L., THOMPSON, D.A. & FRASER, J. (1974).  
The recovery of Pasteurella haemolytica from the tonsils of adult sheep.  
Research in Veterinary Science. 17, 413-414.
- GILMOUR, N.J.L., THOMPSON, D.A., SMITH, W.D. & ANGUS, K.W. (1975).  
Experimental infection of lambs with an aerosol of Pasteurella haemolytica.  
Research in Veterinary Science. 18, 340-341.
- GODING, J.W. (1976).  
Conjugation of antibodies with fluorochromes: modification to the standard methods.  
Journal of Immunological Methods. 13, 215-216.
- GOEBEL, W.F., BINKLEY, F. & PERLMAN, E. (1945).  
Studies on the Flexner group of dysentery bacilli.  
1. The specific antigens of Shigella paradysenteriae (Flexner).  
Journal of Experimental Medicine. 81, 315-330.
- GOLDSCHNEIDER, I., GOTSCHLICH, E.C & ARTENSTEIN, M.S. (1969).  
Human immunity to the meningococcus II. Development of natural immunity.  
Journal of Experimental Medicine. 129, 1327-1348.
- GORSHKOVA, R.P., KOMANDROVA, N.A., KALINOVSKY, A.I., & OVODEV, Y.S. (1980).  
Structural studies on the O specific polysaccharide side chains of Yersinia pseudotuberculosis type III lipopolysaccharide.  
European Journal of Biochemistry. 107, 131-135.

GRAY, B.M. (1979)

ELISA methodology for polysaccharide antigens: Protein coupling of polysaccharides for adsorption to plastic tubes.  
Journal of Immunological Methods. 28, 187-192.

GRIPPENBERG, M., NISSENEN, A., VAISANEN, E. & LINDER, E. (1979).

Demonstration of antibodies against Yersinia enterocolitica lipopolysaccharide in human sera by enzyme-linked immunosorbent assay.

Journal of Clinical Microbiology. 10, 279-284.

HAMDY, A.H., POUNDEN, W.D. & FERGUSON, L.C. (1959).

Microbial agents associated with pneumonia in slaughtered lambs.  
American Journal of Veterinary Research. 20, 87-90.

HANSEN, E.J., FRISCH, C.F., McDADE, R.L.Jnr. & JOHNSTON, K.H. (1981).

Identification of immunogenic outer membrane proteins of Haemophilus influenzae type b in the infant rat model system.

Infection and Immunity. 32, 1084-1092.

HATTEN, B.A. & BRODEUR, R.D. (1978).

Soluble antigens of virulent and attenuated biotypes of Brucella abortus.

Infection and Immununty. 22, (3), 956-962.

HECKELS, J.E. (1977).

The surface of Neisseria gonorrhoeae: isolation of the major components of the outer membrane.

Journal of General Microbiology. 99, 333-341.

HOFF, G.E. & FRASCH, C.E. (1979).

Outer membrane antigens of Neisseria meningitidis group B serotype 2 studied by crossed immunoelectrophoresis.

Infection and Immunity. 25, 849-956.

HOWARD, C.J. & GOURLAY, R.N. (1974).

Electron microscopic examination of certain bovine mycoplasmas stained with ruthenium red and the demonstration of a capsule on Mycoplasma dispar.

Journal of General Microbiology. 83, 393-398.

ITO, J.I., WUNDERLICH, A.C., LYONS, J., DAVIS, C.E., GUINEY, D.G. & BRAUDE, A. (1980).

Role of magnesium in the enzyme linked immunosorbent assay for lipopolysaccharides of rough Escherichia coli strain J5 and Neisseria gonorrhoeae.

Journal of Infectious Diseases. 142, (4), 532-537.

JANN, K. & JANN, B. (1977).

Bacterial polysaccharide antigens, p247-288.

In I.W. Sutherland (ed). Surface carbohydrates of the prokaryotic cell. Academic press, London, New York and San Francisco.



- JANN, K. & JANN, B. (1983).  
The K antigens of Escherichia coli.  
In P. Kallos (ed), Progress in Allergy, Vol 33.  
Karger, Basel.
- JANN, K. & WESTPHAL, O. (1975).  
Microbial polysaccharides, pl-125.  
In M. Sela (ed). The antigens, Vol. 3.  
Academic Press, New York and London.
- JONES, F.S. (1921).  
A study of Bacillus bovisepcticus.  
Journal of Experimental Medicine. 34, 561-577.
- JONES, G.E., GILMOUR, J.S. & RAE, A.G. (1978).  
Endobronchial inoculation of sheep with pneumonic lung-tissue suspensions and with the bacteria and mycoplasmas isolated from them.  
Journal of Comparative Pathology. 88, 85-96.
- JONES, N.C. & OSBORN, M.J. (1977).  
Interaction of Salmonella typhimurium with phospholipid vesicles.  
Journal of Biological Chemistry. 252, 7398-7404.
- JORBECK, H.J.A., SVENSON, S.B. & LINDBERG, A.A. (1981).  
Artificial Salmonella vaccines: Salmonella typhimurium O-antigen-specific oligosaccharide - protein conjugates elicit opsonising antibodies that enhance phagocytosis.  
Infection and Immunity. 32, 497-502.
- KABIR, S., ROSENSTREICH, D.L. & MERGENHAGEN, S.E. (1978).  
Baterial endotoxins and cell membranes, p59-87.  
In J. Jelaszewicz and T. Wadstrom (ed), Bacterial Toxins and Cell Membranes.  
Academic Press, New York and London.
- KARCHE, H. & NIXDORFF, K. (1981).  
Antibody-producing cell responses to an isolated outer membrane protein and to complexes of this antigen with lipopolysaccharide or with vesicles of phospholipids from Proteus mirabilis.  
Infection and Immunity. 31, (3), 862-867.
- KASS, E.H. & WOLFF, M.S.(eds) (1973).  
Bacterial lipopolysaccharides.  
University of Chicago Press. Chicago, London.
- KAUFFMANN, F. (1954).  
Enterobacteriaceae.  
Munksgaard, Copenhagen.
- KEISS, R.E., WILL, D.H. & COLLIER, J.R. (1964).  
Skin toxicity and haemodynamic properties of endotoxin derived from Pasteurella haemolytica.  
American Journal of Veterinary Research. 25, 935-942.

- KNIGHT, H.D., BIBERSTEIN, E.L. & ALLISON, M. (1969).  
The role of capsular and somatic antigens in immunization of mice against P.haemolytica infection.  
Cornell Veterinarian. 59, 55-64.
- KOHLER, G. & MILSTEIN, C. (1975).  
Continuous cultures of fused cells secreting antibody of predefined specificity.  
Nature, (London). 256, 495-497.
- KUCERA, C.J., WONG, C.S. & FELDNER, T.J. (1983).  
Challenge exposure of cattle vaccinated with a chemically altered strain of Pasteurella haemolytica.  
American Journal of Veterinary Research. 44, (10), 1848-1852.
- KUUSI, N., NURMINEN, M., SAXEN, H., VALTONEN, M. & MAKELLA, P.H. (1979).  
Immunization with major outer membrane proteins in experimental salmonellosis of mice.  
Infection and Immunity. 25, 857-862.
- LAM, J.S., GRANOFF, D.M., GILSDORF, J.R. & COSTERTON, J.W. (1980).  
Immunogenicity of outer membrane derivatives of Haemophilus influenzae type b.  
Current Microbiology. 3, 359-364.
- LANDY, M. & BRAUN, W.(eds) (1964).  
Bacterial endotoxins.  
Rutgers University Press. New Brunswick, New Jersey.
- LEIVE, L.L. & DAVIES, B.D. (1980).  
Cell envelope; spores (p73-110).  
In B.D. Davies, R. Dulbecco, H.N. Eisen and H.S. Ginsberg (eds), Microbiology, Third edition. Harper and Row.
- LINDBERG, A.A., HELLERQUIST, C.G., BAGDIAN-MOTTA, G. & MÄKELÄ, P.H. (1978).  
Lipopolysaccharide modification accompanying antigenic conversion by phage p27.  
Journal of General Microbiology. 107, 279-287.
- LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951).  
Protein measurement with folin-phenol reagent.  
Journal of Biological Chemistry. 193, 265-275.
- LUDERITZ, O., GALANOS, C., LEHMANN, V., NURMINEN, M., RIETSCHEL, E.T., ROSENFELDER, G., SIMON, N. & WESTPHAL, O. (1973).  
Lipid A: Chemical structure and biological activity.  
Journal of Infectious Diseases. 128, suppl, 17-29.
- LUDERITZ, O., STAUB, A.M. & WESTPHAL, O. (1966).  
Immunochemistry of O and R antigens of Salmonella and related Enterobacteriaceae.  
Bacteriological Reviews. 30, 192-255.

LÜDERITZ, O., WESTPHAL, O., STAUB, A.M. & NIKAIDO, H. (1971).  
Isolation and chemical and immunological characterisation of  
bacterial lipopolysaccharides, pl45-233.  
In G. Weinbairn, S. Kadis and S.J. Ajl (eds). Microbial Toxins  
volIV. Academic Press. Inc., London.

MAHESWARAN, S.K., BERGGREN, K.A., SIMONSON, R.R., WARD, G.E. &  
MUSCOPLAT, C.C. (1980).  
Kinetics of interaction and fate of Pasteurella haemolytica in  
bovine alveolar macrophages.  
Infection and Immunity. 30, (1), 254-262.

MANEVAL, W.E. (1941).  
Staining bacteria and yeasts with acid dyes.  
Stain Technology. 16, 13-19.

McCABE, W.R., BRUINS, S.E., CRAVEN, D.E. & JONS, M. (1977).  
Cross reactive antigens: Their potential for  
immunization-induced immunity to Gram-negative bacteria.  
Journal of Infectious Diseases. (supplement) 136: S161-S166.

McKERCHER, D.G. (1978).  
Disease incidence and epidemiology - The situation in the U.S.A.  
p71-83.  
In W.B., Martin, (ed), Respiratory diseases in cattle.  
Martinus Nijhoff, The Hague.

MILES, A.A., MISRA, S.S. & IRWIN, J.O. (1938).  
The estimation of the bactericidal power of the blood.  
Journal of Hygiene, Cambridge. 38, 732-749.

MINISTRY of AGRICULTURE, FISHERIES and FOOD. (1964)  
Report No.3, Animal disease survey.  
London, H.M. Stationary Office.

MIURA, T. & MIZUSHIMA, S. (1968).  
Separation by density gradient centrifugation of two types of  
membranes from spheroplast membrane of Escherichia coli K12.  
Biochemica et Biophysica Acta. 150, 159-161.

MIURA, T. & MIZUSHIMA, S. (1969).  
Separation and properties of outer and cytoplasmic membranes in  
Escherichia coli.  
Biochemica et biophysica Acta. 193, 268-276.

MOMOI, M., KENNET, R.H. & GLICK, M.C. (1980).  
A membrane glycoprotein from human neuroblastoma cells isolated  
with the use of a monoclonal antibody.  
Journal of Biological Chemistry. 255, (24), 11914-11921.

MONTGOMERIE, R.F., BOSWORTH, T.G. & GLOVER, R.E. (1938).  
Enzootic pneumonia in sheep.  
Journal of Comparative Pathology. 51, 87-107.



- MORRISON, D.C. & LEIVE, L. (1975).  
Fractions of lipopolysaccharide from Escherichia coli 0111:B4 prepared by two extraction procedures.  
Journal of Biological Chemistry. 250, 2911-2919.
- MUHLRADT, P.F. & GOLECKI, J.R. (1975).  
Asymmetrical distribution and artifactual reorientation of lipopolysaccharide in the outer membrane bilayer of Salmonella typhimurium.  
European Journal of Biochemistry. 51, 343-352.
- MUKKUR, T.K.S. (1977).  
Demonstration of cross protection between Pasteurella multocida type A and Pasteurella haemolytica serotype 1.  
Infection and Immunity. 18, 583-585.
- NAKAE, T. (1976).  
Identification of the outer membrane protein of Escherichia coli that produces trans-membrane channels in reconstituted vesicle membranes.  
Biochemical and Biophysical Research Communications. 71, 877-884.
- NEWSOM, I.F. & CROSS, F. (1932).  
Some bipolar organisms found in pneumonia of sheep.  
Journal of the American Veterinary Medicine Association. 80, 711-719.
- NIKAIDO, H. & NAKAE, T. (1979).  
The outer membrane of Gram-negative bacteria.  
Advances in Microbial Physiology. 20, 163-250.
- NOWOTNY, A. (1966).  
Heterogenicity of endotoxic bacterial lipopolysaccharides revealed by ion exchange chromatography.  
Nature, (London). 210, 278-280.
- OGATA, S., KANAMORI, M. & MIYASHITA, K. (1972).  
Antigenicity of protein and lipopolysaccharide from Yersinia enterocolitica.  
Contributions to Microbiology and Immunology 5, 64-72.
- OLITZKI, L. (1948).  
Mucin as a resistance lowering substance.  
Bacteriology Reviews. 12, 149-172.
- OLMOS, A. & BIBERSTEIN, E.L. (1979).  
Differentiation of P.haemolytica biotypes A and T with growth inhibitors.  
Journal of Clinical Microbiology. 10, 231-234.
- ORSKOV, I., ORSKOV, F., JANN, B. & JANN, K. (1977).  
Serology, chemistry and genetics of O and K antigens of Escherichia coli.  
Bacteriology Reviews. 41, 667-710.

OSBORN, M.J. (1963).

Studies on the Gram-negative cell wall: I. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of Salmonella typhimurium.

Biochemistry. 50, 499-500.

OSBORN, M.J., GANDER, J.E., PARIS, E. & CARSON, J. (1972).

Mechanisms of assembly of the outer membrane of Salmonella typhimurium: isolation and characterisation of cytoplasmic membrane and outer membrane.

Journal of Biological Chemistry. 247, 3962-3972.

OUCHTERLONY, O. (1948).

In vitro method for testing the toxic producing capacity of diphtheria bacteria.

Scandinavia. 25, 186-191.

PALMER, J.W. & GERLOUGH, T.D. (1940)

A simple method for preparing antigenic substances from the typhoid bacillus.

Science. 92, 155-156.

PEGRAM, R.G., ROEDER, P.L. & SCOTT, J.M. (1979).

Two new serotypes of Pasteurella haemolytica from sheep in Ethiopia.

Tropical Animal Health and Production. 11, 29-30.

PENN, C.W. & NAGY, L.K. (1976).

Isolation of a protective, non toxic capsular antigen from Pasteurella multocida, types B and E.

Research in Veterinary Science. 20, 90-96.

POXTON, I.R. (1979).

Serological identification of Bacteroides species by an ELISA.

Journal of Clinical Pathology. 32, 294-298.

POXTON, I.R. & BROWN, R. (1979).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of cell surface proteins as an aid to the identification of Bacteroides fragilis group.

Journal of General Microbiology. 112, 211-217.

POXTON, I.R. & IP, K-Y. M. (1981).

The cell-surface antigens of Bacteroides vulgatus.

Journal of General Microbiology. 126, 103-109.

POXTON, I.R. & SUTHERLAND, I.W. (1976).

Isolation of rough mutants of Klebsiella aerogenes and their synthesis of polysaccharides.

Journal of General Microbiology. 96, 195-202.

- RAZIUDDIN, S. (1980).  
Immunochemical studies of the lipopolysaccharides of Vibrio cholerae. Constitution of O specific side chain and core polysaccharide.  
Infection and Immunity. 27, 211-215.
- REAVELY, D.A. & BURGE, R.E. (1972).  
Walls and membrane in bacteria.  
Advances in microbial physiology. 7, 1-81.
- REED, L.J. & MUENCH, H. (1938).  
A simple method for estimating 50% end points.  
American Journal of Hygiene. 27, 493-497.
- RIMSAY, R.L., COYLE-DENNIS, J.E., LAUERMAN, L.H. & SQUIRE, P.G. (1981).  
Purification and biological characterisation of endotoxin fractions from Pasteurella haemolytica.  
American Journal of Veterinary Research. 42, 2134-2138.
- ROGERS, H.J., PERKINS, H.R. & WARD, J.B. (eds) (1981).  
Isolation of walls and membranes p45-71. In Microbial cell walls and Membranes.  
Chapman and Hall. London.
- ROITT, I.M. (1980).  
Essential Immunology. Third edition.  
Blackwell Scientific Publications.  
Oxford, London, Edinburgh, Melbourne.
- ROWLEY, D. (1971).  
Endotoxins and bacterial virulence.  
Journal of Infectious Disease. 123, 317-327.
- RUSHTON, B. (1978).  
Induction of pneumonia in mice with Pasteurella haemolytica.  
Journal of Comparative Pathology. 88, 477-480.
- RUSSELL, H., EDWARDS, L.R., WORTHAM, E.W. & FACKLAM, R.R. (1980).  
Enzyme linked immunosorbent assay for detection of antibodies against Streptococcus pneumoniae capsular polysaccharide.  
Journal of Clinical Microbiology. 11, (2), 198-199.
- SCHMERR, M.J.F. & REBERS, P.A. (1979).  
Studies of immunogenicity of Westphal lipopolysaccharides from Pasteurella multocida in mice.  
Journal of Comparative Pathology. 89, 531-539.
- SCHMIDT, G., JANN, B. & JANN, K. (1969).  
Immunochemistry of R lipopolysaccharides of Escherichia coli.  
European Journal of Biochemistry. 10, 501-510.
- SCHNAITMAN, C.A. (1971).  
Solubilisation of the cytoplasmic membrane of Escherichia coli by Triton X100.  
Journal of Bacteriology. 108, 545-552.

SCHNEERSON, R., BARRERA, O., SUTTON, A. & ROBBINS, J.B. (1980a). Preparation, characterization and immunogenicity of Haemophilus influenzae type b polysaccharide protein conjugates. Journal of Experimental Medicine. 152, 361-376.

SCHNEERSON, R., ROBBINS, J.B., BARRERA, O., SUTTON, A., HABIG, W.B., HARDEGREE, M.N. & CHAIMOVICH, J. (1980b). Haemophilus influenzae type b polysaccharide-protein conjugates: model for a new generation of capsular polysaccharide vaccines. Progress in Clinical and Biological Research. 47, 77-94.

SCHURIG, G.G., JONES, L.M., SPETH, S.L. & BERMAN, D.T. (1978). Antibody response to antigens distinct from smooth lipopolysaccharide complex in Brucella infection. Infection and Immunity. 21, 994-1002.

SHARP, J.M., GILMOUR, N.J.L., THOMPSON, D.A. & RUSHTON, B. (1978). Experimental infection of specific pathogen free lambs with para influenza virus type 3 and Pasteurella haemolytica. Journal of Comparative Pathology. 88, 237-243.

SHIRAI, A., DIETAL, J.W. & OSTERMAN, J.V. (1975). Indirect haemagglutination test for human antibody to typhus and spotted fever groups rickettsiae. Journal of Clinical Microbiology. 2, 430-437.

SHREEVE, B.J., BIBERSTEIN, E.L. & THOMPSON, D.A. (1972). Variation in carrier rates of Pasteurella haemolytica in sheep. II. Diseased flocks. Journal of Comparative Pathology. 82, 111-116.

SJEWICZYK, B. & TAYLOR, A. (1980). Diversity of Vi related antigens in the microcapsule of Salmonella typhi. Infection and Immunity. 30, 661-667.

SMIT, J., KAMINO, Y. & NIKAIDO, H. (1975). Outer membrane of Salmonella typhimurium: Chemical analysis and freeze-fracture studies with lipopolysaccharide mutants. Journal of Bacteriology. 942-958.

SMITH, G.R. (1959a). Pasteurella haemolytica: A study of pathogenicity and immunity in laboratory animals and sheep. PhD Thesis, University of Edinburgh.

SMITH, G.R. (1959b). Isolation of two types of Pasteurella haemolytica from sheep. Nature (London). 183, 1132-1133.

SMITH, G.R. (1959c). Pasteurella haemolytica: A study of pathogenicity and immunity in laboratory animals and sheep. PhD Thesis, University of Edinburgh.

SMITH, G.R. (1961).

The characteristics of two types of Pasteurella haemolytica associated with different pathological conditions in sheep.  
Journal of Pathological Bacteriology. 81, 431-440.

SMITH, H. (1977).

Microbial surfaces in relation to pathogenicity.  
Bacteriological Reviews. 41, 475-500.

SMITH, J.E. & THAL, E. (1965).

A taxonomic study of the genus Pasteurella using a numerical technique.

Acta Pathologica et Microbiologica Scandinavica. 64, 213-223.

SMITH, W.D., DAWSON, A.McL., WELLS, P.W. & BURRELLS, C. (1975).

Immunoglobulin concentrations in ovine body fluids.

Research in Veterinary Science. 19, 189-194.

SMYTH, C.J., SIEGEL, J., SALTON, M.R.J. & OWEN, P. (1978).

Immunochemical analysis of inner and outer membranes of Escherichia coli by crossed immunoelectrophoresis.

Journal of Bacteriology. 133, 306-319.

SNEDECOR, G.W. & COCHRAN, W.G. (1967).

Statistical methods. 6th edition.

Iowa State University Press, Ames, Iowa, USA.

STAMP, J.T., WATT, J.A.A. & THOMLINSON, J.R. (1955).

Pasteurella haemolytica septicaemia of lambs.

Journal of Comparative Path. 65, 183-196.

STEELE, E., JENKIN, C.R. & ROWLEY, D. (1977).

Protective antigens of bacteria, p247-284.

In M. Sela (ed), The Antigens. Academic Press, New York and London.

STEVENS, A.J. (1957).

Respiratory diseases of sheep.

Veterinary Record. 69, 1249-1254.

STEVENSON, R.G. (1974).

Streptococcus zooepidemicus infection in sheep.

Canadian Journal of Comparative Medicine. 38, 243-250.

SUTHERLAND, A.D., GRAY, E. & WELLS, P.W. (1983)

Cytotoxic effect of Pasteurella haemolytica on ovine bronchoalveolar macrophages in vitro.

Veterinary Microbiology. 8, 3-15.

SUTHERLAND, I.W.(ed)(1977).

Bacterial exopolysaccharides - their nature and composition. p27-96.

Surface carbohydrates of the prokaryotic cell.

Academic Press, London, New York, San Francisco.



SVENDSEN, P.J. (1973).

Fused rocket immunoelectrophoresis.

Scandinavian Journal of Immunology. 2, suppl 1, 69-70.

SVENSON, S.B., NURMINEN, M. & LINDBERG, A.A. (1979).

Artificial Salmonella vaccines: O antigenic oligosaccharide - protein conjugates induce protection against infection with Salmonella typhimurium.

Infection and Immunity. 25, 863-872.

TADAYON, R.A. & LAUERMAN, L.H. (1981).

The capacity of various fractions of Pasteurella haemolytica to stimulate protective immunity in mice and hamsters.

Veterinary Microbiology. 6, 85-93.

TAPLITS, M. & MICHAEL, J.G. (1979).

Immune response to E.coli B surface antigens.

Infection and Immunity. 25, (3), 943-945.

THOMPSON, D.A. (1973).

Experimental studies on Pasteurella haemolytica.

M.Phil. Thesis, University of Edinburgh.

THOMPSON, D.A. & MOULD, D.L. (1975).

Protein electrophoretic pattern of Pasteurella haemolytica.

Research in Veterinary Science. 18, 342-343.

THOMPSON, D.A., FRASER, J. & GILMOUR, N.J.L. (1977).

Serotypes of Pasteurella haemolytica in ovine pasteurelosis.

Research of Veterinary Science. 22, 130-131.

TOWBIN, H., STACHELIN, T. & GORBIN, J. (1979).

Electrophoretic transfer of proteins from acrylamide gels to nitro cellulose sheets: procedure and some applications.

Proceedings of the National Academy of Sciences of the United States of America. 76, 4350-4354.

TREVELYAN, W.E., PROCTER, D.P. & HARRISON, J.S. (1950).

Detection of sugars on paper chromatograms.

Nature, London. 166, 444-445.

VAN DER VEEN, R.R. & ZUMPT, I.F. (1967).

Enzootic pneumonia of sheep in the Mafeking district.

Journal of the South African Veterinary Medical Association. 38, 415-419.

Veterinary investigation diagnosis analysis II report. (1977).

Ministry of Agriculture, Fisheries and Food and Department of Agriculture and Fisheries for Scotland.

VIRJI, M., HECKELS, J.E. AND WATT, P.J. (1983).

Monoclonal antibodies to gonococcal pili: Studies on antigenic determinants on pili from variant of strain P9.

Journal of General Microbiology. 129, 1965-1973.

- WEEKE, B. (1973a).  
General remarks on principles, equipment reagents and procedures.  
Scandinavian Journal of Immunology. 2, suppl 1, 15-35.
- WEEKE, B. (1973b).  
Crossed immunoelectrophoresis.  
Scandinavian Journal of Immunology. (2) Suppl 1. 47-56.
- WEI, B.D. & CARTER, G.R. (1978).  
Live streptomycin-dependent Pasteurella multocida vaccine for the prevention of hemorrhagic septicaemia.  
American Journal of Veterinary Research. 39, (9), 1534-1537.
- WEIDEL, W., FRANK, H. & MARTIN, H.H. (1960).  
The rigid layer of the cell wall of Escherichia coli strain B.  
Journal of General Microbiology. 22, 158-166.
- WEISS, R., SCHIEFER, H.V. & KRAUSS, H. (1979).  
Ultrastructure visualization of Klebsiella capsules by polycationic ferritin.  
FEMS Microbiology letters. 6, 435-437.
- WELLS, P.W., GILMOUR, N.J.L., BURRELLS, C. & THOMPSON, D.A. (1979).  
A serological comparison of P.haemolytica vaccines containing different adjuvants.  
Research in Veterinary Science. 27, 248-250.
- WESSMAN, G.E. (1965).  
Cultivation of Pasteurella haemolytica in a casein hydrolysate medium.  
Applied Microbiology. 13, (3), 426-431.
- WESTPHAL, O. (1975).  
Bacterial endotoxins.  
International Archives of Allergy and Applied Immunology. 49, 1-43.
- WESTPHAL, O., LUDERITZ, O. & BISTER, F. (1952).  
Über die extraction von bakterien mit phenol/wasser.  
Zeitschrift für Naturforschung. 76, 144-145.
- WILKINSON, S.G. (1977).  
Composition and structure of bacterial lipopolysaccharides. p97-106.  
In I.W. Sutherland (ed), Surface carbohydrate of the prokaryotic cell. Academic Press, London, New York and San Francisco.
- WINKLE, S., REFAI, M. & ROHDE, R. (1972).  
On the antigenic relationship of Vibrio cholerae to Enterobacteriaceae.  
Annales de L'Institut Pasteur, Paris. 123, 775-781.

WINTER, A.J., McCOY, E.C., FULLMER, C.S., BURDA, K. & BIER, P.J. (1978).

Microcapsule of Campylobacter fetus: chemical and physical characterisation.

Infection and Immunity. 22, (3), 963-971.

WOLF-WATZ, H., NORMARK, S. & BLOOM, G.D. (1973).

Rapid method for isolation of large quantities of outer membrane from Escherichia coli K12 and its application to the study of envelope mutants.

Journal of Bacteriology. 115, 1191-1197.

WRIGHT, A. & KANEGASAKI, S. (1971).

Molecular aspects of lipopolysaccharides.

Physiological Reviews. 51, 748-784.

YATES, W.D.G. (1982).

A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle.

Canadian Journal of Comparative Medicine. 46, 225-263.



Publications arising from this thesis

DONACHIE, W. & JONES, G.E. (1982)

The use of ELISA to detect antibodies to Pasteurella haemolytica A2 and Mycoplasma ovipneumoniae in sheep with experimental chronic pneumonia. (p102-111)

In Wardley, R.C. and Crowther, J.R. (eds), The ELISA: enzyme-linked immunoabsorbent assay in veterinary research and diagnosis. Current Topics in Veterinary Medicine and Animal Science Vol 22 Martinus Nijhoff, The Hague/Boston/London.

DONACHIE, W., BURRELLS, C. & DAWSON, A. McL. (1983)

Specificity of the enzyme-linked immunosorbent assay (ELISA) for antibodies in the sera of specific pathogen-free lambs vaccinated with Pasteurella haemolytica antigens

Veterinary Microbiology. 8, 199-205.

DONACHIE, W., GILMOUR, N.J.L., MOULD, D.L. & POXTON, I.R. (1984)

Comparison of cell surface antigen extracts from two serotypes of Pasteurella haemolytica.

Journal of General Microbiology. (In press).

THE USE OF ELISA TO DETECT ANTIBODIES TO PASTEURELLA HAEMOLYTICA A2  
AND MYCOPLASMA OVIPNEUMONIAE IN SHEEP WITH EXPERIMENTAL CHRONIC  
PNEUMONIA

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ABSTRACT

Ten untreated sheep and 18 sheep in which chronic pneumonia had been experimentally reproduced were monitored for serum antibodies to Pasteurella haemolytica serotype A2 and Mycoplasma ovipneumoniae by ELISA tests. The pasteurella test utilised a phenol water extract of P. haemolytica A2 cells as antigen: for the mycoplasma test whole M. ovipneumoniae cells solubilised in a carbonate:bicarbonate buffer were used.

A rise in antibody titre in the infected animals was detected at 2 weeks post inoculation (wpi) and a peak in mean titres at 8 wpi for P. haemolytica and 14 wpi for M. ovipneumoniae. Titres to both organisms declined slowly thereafter till termination of the experiment at 24.5 wpi.

An indirect haemagglutination (IHA) test for P. haemolytica serotype A2 antibodies demonstrated a similar pattern of antibody rise and decline, but at considerably lower titres than provided by the ELISA test.

It was concluded that the ELISA tests for both P. haemolytica and M. ovipneumoniae were sensitive and have potential application in the detection of antibody responses in ovine respiratory disease.

INTRODUCTION

Chronic ovine (atypical) pneumonia is a disease of lambs up to 1 year old (Stamp and Nisbet, 1963). Mycoplasma ovipneumoniae and Pasteurella haemolytica A biotypes are associated with the disease in the field (Jones et al, 1979) and inoculation of pneumonic lesion homogenates containing both organisms induces a proliferative exudative (PE) pneumonia indistinguishable from the naturally occurring condition (Jones et al, 1978). Clinical signs of atypical pneumonia are frequently mild or inapparent, yet its effect on growth and production may be severe (Jones et al, in preparation). It was thus considered important to develop methods to assist the detection of atypical pneumonia and to this end we have investigated the value of ELISA tests for antibodies to both P. haemolytica and M. ovipneumoniae.

Burrells et al (1979) used an antigen prepared by sodium salicylate extraction (SSE) of P. haemolytica cells and found their ELISA much more sensitive than the standard indirect haemagglutination (IHA) test (Biberstein et al, 1960) for the measurement of antibody to

P. haemolytica. Subsequently, however, the SSE antigen has been shown to contain antigens common to all P. haemolytica serotypes resulting in ELISA cross reactions (Burrells, unpublished observations). In this paper we report ELISA results obtained with an antigen prepared by phenol-water extraction of P. haemolytica biotype A, serotype 2 (A<sub>2</sub>).

Several different methods have been used to prepare mycoplasma antigens for use in ELISA tests, including solubilization in carbonate: bicarbonate buffer (Horowitz and Cassell, 1978), in sodium dodecyl sulphate (Bruggman et al, 1977), and in Tween 20 followed by gel filtration (Nicolet et al, 1981). Initial trials with M. ovipneumoniae antigen suspensions treated according to the method of Bruggman et al (1977) indicated that the antigen produced was sensitive but labile, even after storage at -20°C (Jones, unpublished observations). Whole cell suspensions treated according to the method of Horowitz and Cassell (1978) were found to be stable if stored at -20°C; their performance as ELISA antigens is described in this communication.

## MATERIALS AND METHODS

### Antigens

**Mycoplasma:** Preparation of washed, whole cell suspensions of strain 956/2 of M. ovipneumoniae was as previously described (Jones et al, 1976), except that the medium used was preincubated and shaken at 37°C for 2 days, then filtered through 0.22 µm average pore diameter (a.p.d.) membrane filters (Millipore UK, Harrow, Middlesex) before use. The cell suspensions were analysed for protein content by a semi-micro Kjeldahl method then solubilized in a carbonate: bicarbonate buffer (CBB) according to the method of Horowitz and Cassell (1978). Aliquots of the suspension were stored at -20°C until use, when they were diluted to provide 5 µg protein/ml.

**Bacteria:** P. haemolytica A<sub>2</sub> cells were grown in nutrient broth (Oxoid No. 2) for 18 h at 37°C. Cells were harvested by centrifugation at 4000 g for 30 minutes then washed twice in distilled water. A phenol water extract (PWE) was prepared using the method of Westphal et al, (1952). Ten grammes wet weight of cells were resuspended to 50 ml in distilled water and heated to 68°C. An equal volume of 90% phenol at 68°C was added to the suspension and the mixture kept at 68°C for 10 minutes with vigorous shaking. The

mixture was quickly cooled to 4°C on an ice bath then centrifuged at 9000 g for 30 minutes. The aqueous layer was removed, dialysed against running water for 24 hours then concentrated twofold by rotary evaporation before centrifugation at 100,000 g for 2 h. The deposit, resuspended in 5 ml distilled water, was recentrifuged and the resultant pellet taken up in 1 ml distilled water and lyophilised. A concentration of 2 mg/ml in distilled water was used in the ELISA test.

#### Experimental design

Twenty eight lambs, approximately 10 weeks old, were obtained from a flock in which levels of infection with potentially pathogenic respiratory microorganisms had been found to be very low over 4 years of sampling. Nasal swabs taken from the lambs on day 0 were free of *pasteurellae*, *mycoplasmas* and viruses.

Eighteen lambs were inoculated intratracheally on day 0 with 6 ml of a homogenate of pneumonic lung lesions obtained from the abattoir (Jones *et al*, 1978). This homogenate contained  $10^6$  colour changing units (ccu) per 0.2 ml of *M. ovipneumoniae* and  $2 \times 10^3$  colony forming units (cfu) of *P. haemolytica* A2 with no isolation of other microorganisms. The same animals were inoculated intranasally on day 7 with 1 ml of a culture of *P. haemolytica* A2 containing  $4 \times 10^6$  cfu/ml. The remaining 10 control lambs were untreated throughout. All control lambs and 6 infected lambs were slaughtered at 15.5 wpi; the surviving 8 infected lambs were slaughtered at 24.5 wpi, when they had reached the same mean liveweight as the controls.

#### Sera

##### Standard *M. ovipneumoniae* anti serum:

A vaccine incorporating an aluminium hydroxide adsorbed suspension of strain 956/2 mixed in equal proportions with Bayol and Arlacel (Jones 1978) was administered subcutaneously twice with a 2 week interval to two 3-week old gnotobiotic lambs. The lambs were killed and bled out 7 days after the second vaccination. The sera from both animals were pooled and stored in aliquots at -20°C until used at dilutions between 1/2000 and 1/256,000.

##### Standard *P. haemolytica* anti-serum:

Sera from sheep shown to have antibody to *P. haemolytica* A2 in a previous experiment were pooled and stored in aliquots at -20°C until used at dilutions between 1/2000 and 1/256,000.

## Serology

IHA test: this was carried out as described by Fraser et al (in press).

ELISA tests: the methods used were based on that described by Engvall and Perlman (1972). The tests consisted of 5 steps with 3 washes of 0.05% Tween 20 in phosphate buffered saline pH 7.4 (PBS/Tween) after each step and 200  $\mu$ l volumes of reagents, at pre-determined optimal dilutions, were used throughout.

For P. haemolytica the steps were: I. Micro ELISA plates (M192A Dynatech Laboratories Ltd., - Billinghamurst, Sussex) were coated with PWE antigen diluted 1/200 in carbonate bicarbonate buffer pH 9.6. The plates were incubated at 37°C for 24 hr then held at 4°C for 7 days.

II. Standard or test sera were added to all wells except those in column 1 to which diluent was added. Test sera were diluted 1/2000 in PBS/Tween + 0.02% sodium azide before use. The standard serum was made up in 8 two fold dilutions between 1/2000 and 1/256,000. Each sample (test and different dilutions of standard) was tested in duplicate and the plates incubated at room temperature (RT) for 3 h.

III. Pig IgG anti-sheep IgG conjugated with alkaline phosphatase was added to each well and incubated for 3 h at RT.

IV. Substrate (p-nitrophenyl phosphate, Sigma Chemical Co. Poole, Dorset; 1 mg/ml) was added to each well and incubated at RT for 18 h.

V. 3 M NaOH was added to stop the reaction. The results were recorded as optical densities at a wavelength of 405 nm ( $OD_{405}$ ) using an automatic multichannel spectrophotometer ("Titertek Multiskan", Flow Laboratories, Irvine, Ayrshire).

The steps for M. ovispneumoniae were as follows:

I. The antigen was added to each well and incubated at RT overnight.

II. The standard and test sera were dispensed as for the P. haemolytica ELISA but the plates were incubated for 4 hours at 37°C.

III. Conjugate (as above) was added to all wells and incubated overnight at RT.

IV. Substrate (as above) was added to all wells and incubated for 1 hr at 37°C.

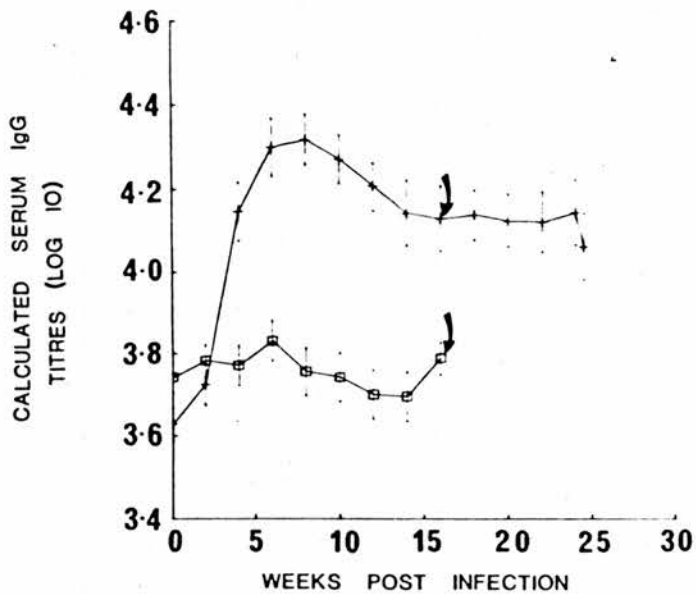


Figure 1. Mean calculated serum antibody titres ( $\log_{10}$ ) against P. haemolytica A2 as detected by ELISA for infected group (+) and control group (□). Arrow indicates time of first killing at 15.5 wp.i.

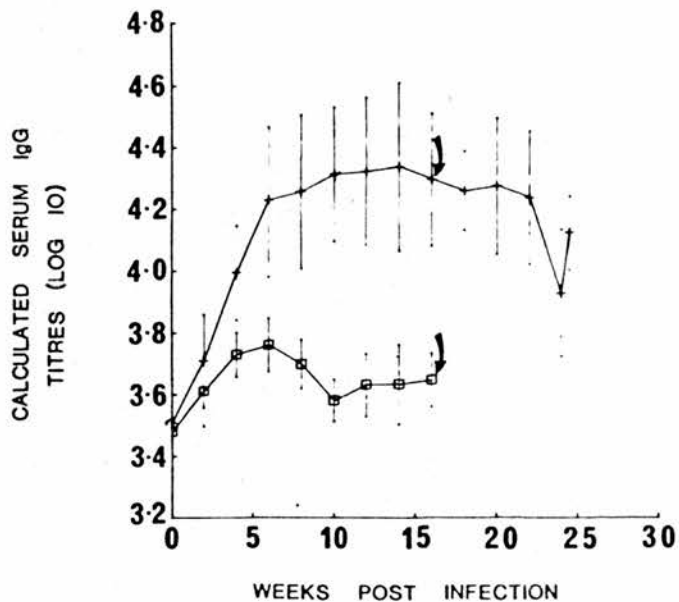


Figure 2. Mean calculated serum antibody titres ( $\log_{10}$ ) against M. ovipneumoniae as detected by ELISA for infected group (+) and control group (□). Arrow indicates time of first killing at 15.5 wpi.

V. As for P. haemolytica ELISA.

#### Calculation of results

1. The absorbance figures (A) were converted to  $\log_{10}$  values.
2. The  $\log A$  values of the standard serum dilutions were plotted against the  $\log_{10}$  values of these dilutions to provide a standard graph.
3. The titre of the standard serum was taken to be that dilution which would give an absorbance value of 0.1 ( $\log 1.0$ ) as negative control sera from SPF and gnotobiotic lambs were never found to exceed this value.
4. The titres of the test sera are calculated from the formula

$$\begin{array}{lcl} \text{titre of test} & & \text{Reciprocal of dilution of} \\ \text{serum} & = & \frac{\text{test serum (2000)}}{\text{Reciprocal of dilution of}} \times \text{titre of standard} \\ & & \text{standard serum which would} \\ & & \text{give the same A value as the} \\ & & \text{test serum (read from graph)} \end{array}$$

#### RESULTS

ELISA. The calculated mean serum antibody titres against P. haemolytica and M. ovipneumoniae for infected and control animals are shown in Figures 1 and 2 respectively. Mean antibody titres against P. haemolytica in the infected group peaked at 8 wpi (1/27,500) while antibody titres against M. ovipneumoniae peaked at 14 wpi (1/21,480).

Antibodies against P. haemolytica A2 declined quickly after peaking while those against M. ovipneumoniae persisted at a high level until 24 wpi. The control animals, in both tests, showed no significant rise in mean antibody titre over the duration of the experiment.

IHA - The mean serum antibody titres of the infected group obtained in the IHA test plotted against time are shown in Figure 3. Antibody detected in this test increased quickly to a titre of 1/48 at 4 wpi and declined sharply thereafter. The mean serum antibody titres of the controls remained almost constant (at 1 in 2) throughout the experiment.

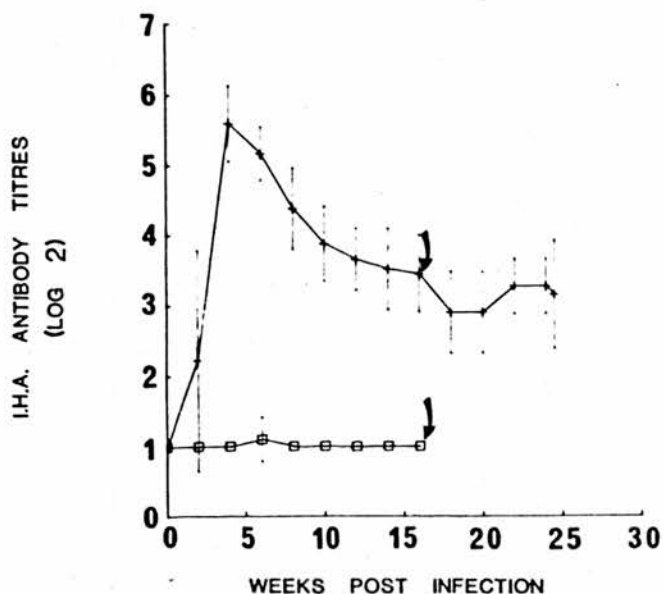


Figure 3. Mean serum antibody titre ( $\log_2$ ) against P. haemolytica as detected by IHA for infected group (+) and control group (□). Arrow indicates time of first killing at 15.5 wpi.

Necropsy - The principal pathological and microbiological findings are shown in Table 1. PE pneumonia was detected in all infected animals but not in any control animal. Lungs from all infected animals and 3 controls yielded M. ovipneumoniae; P. haemolytica was recovered from the lungs of 2 infected animals killed at 15.5 wpi.

Table 1. Principal pathological and microbiological findings in infected and control groups at necropsy.

Group	No. in group	No. with PE pneumonia	No. positive for	
			<u>M. ovipneumoniae</u>	<u>P. haemolytica</u>
Infected	14	14	14	2
Control	9	0	3	0



## DISCUSSION

Sheep in which pneumonia had been experimentally reproduced with suspensions containing P. haemolytica and M. ovipneumoniae showed rises in antibody titre to these two organisms by ELISA tests. Group mean titres to the bacterium rose more quickly than those to the mycoplasma, peaking at 8 weeks for the former and 14 weeks for the latter. Reasons for this difference are uncertain but may involve a greater invasiveness or antigenicity of the bacterium compared with the mycoplasma. One possibility, that the response to P. haemolytica was anamnestic and that to M. ovipneumoniae was primary, is a less likely explanation since low levels of antibody to both organisms appeared to be present in pre-inoculation sera, suggesting prior exposure of the animals to the two agents. Whatever the cause, the ELISA test for P. haemolytica would seem to be more useful diagnostically in the early phases of PE pneumonia. The relatively rapid decline in titres to P. haemolytica compared with M. ovipneumoniae probably reflects the early elimination of the bacterium and the lengthy persistence of the mycoplasma in lesions of PE pneumonia (Gilmour et al, in press), suggesting that the ELISA test for M. ovipneumoniae antibodies may be more useful diagnostically in the subacute phase of the disease.

None of the 9 control sheep showed significant rises in antibody titre to pasteurilla or mycoplasma above the levels present at the start of the experiment, despite the isolation of M. ovipneumoniae from the lungs of 3 sheep at slaughter. This finding corroborates earlier observations (Jones, unpublished observations) that simple pulmonary colonisation with M. ovipneumoniae is insufficient to induce seroconversion, and that lung consolidation is necessary before significant rises in antibody are detected.

Phenol-water extracts of serotypes of P. haemolytica have been shown to contain the serotype specific antigen for that serotype (Donachie, unpublished observations). This extract contains lipopolysaccharide (Wilkinson, 1977) and as P. haemolytica serotypes may share antigenic determinants in this component this may decrease the specificity of the ELISA test and contribute to the "background" level of antibody detected. Difficulty in binding polysaccharide antigens to polystyrene has been noted previously (Barret et al, 1980). This

problem was overcome in these studies by incorporation of a prolonged binding step (Dahlberg et al, 1980) of 24 h incubation at 37°C followed by 7 days at 4°C.

The IHA test was less sensitive than the ELISA, but demonstrated maximum titres at an earlier stage, suggesting that a different antibody was assayed. The IHA test measures only antibodies to those antigens which have affinity for erythrocyte membrane sites while all antigens binding to the well are available for antibody-antigen reactions in the ELISA test.

The ELISA test for M. ovipneumoniae antibodies used whole cell antigen solubilized in CBB and proved extremely sensitive in these studies. However, this antigen has been found to give positive results with hyperimmune sera to a variety of mycoplasma species (Jones, unpublished observations) and it may prove necessary to develop a more specific antigen for future use.

In conclusion it would seem that the ELISA has potential for serodiagnosis of pneumonia, as the combination of high serum antibody titres to both P. haemolytica and M. ovipneumoniae which are easily detected by the assay, are correlated with consolidated lesions. However a more specific antigen for both assays would give greater confidence in the test and make it a more powerful diagnostic tool.

## REFERENCES

- Barret, J.D., Amman, A.J., Stenmark, S. and Wara, D.W. (1980). Immunoglobulin G and M antibodies to pneumococcal polysaccharide detected by enzyme-linked immunosorbent assay. *Inf. & Immun.* 27, 411-417.
- Biberstein, E.L., Gills, M. and Knight, H. (1960). Serological types of Pasteurella haemolytica. *Cornell Vet.* 50, 223-300.
- Bruggman, S., Keller, H., Bertschunger, H.U. and Engberg, G. (1977). Quantitative detection of antibodies to Mycoplasma suipneumoniae in pigs' sera by an enzyme-linked immunosorbent assay. *Vet. Record*, 101, 109-111.
- Burrells, C., Wells, P.W. and Dawson, A.McL. (1979). The quantitative estimation of antibody to Pasteurella haemolytica in sheep using a micro enzyme-linked immunosorbent assay (ELISA). *Vet. Microbiol.* 3, 291-301.
- Dahlberg, T. and Branefors, P. (1980). Enzyme-linked immunosorbent assay for titration of Haemophilus influenzae capsular and O antigen antibodies. *J. clin. Micro.* 12, 185-192.
- Fraser, J., Laird, S. and Gilmour, N.J.L. (1982). A new serotype (Biotype T) of Pasteurella haemolytica. *Res. vet. Sci.* (in press).
- Gilmour, J.S., Jones, G.E., Keir, W.A. and Rae, A.G. Long-term pathological and microbiological progress in sheep of experimental disease resembling atypical pneumonia. *J. comp. Path.* (in press).
- Horowitz, S.A. and Cassell, G.A. (1978). Detection of antibodies to Mycoplasma pulmonis by an enzyme linked immunosorbent assay. *Inf. & Immun.* 22, 161-170.
- Jones, G.E., Foggie, A., Mould, D.L. and Livitt, S. (1976). The comparison and characterisation of glycolytic mycoplasmas isolated from the respiratory tract of sheep. *J. med. Microbiol.* 9, 39-51.
- Jones, G.E. (1978). Studies on mycoplasmas of the respiratory tract of sheep. PhD. Thesis University of Edinburgh.
- Jones, G.E., Gilmour, J.S. and Rae, A.G. (1978). Endobronchial inoculation of sheep with pneumonic lung-tissue suspensions and with the bacteria and mycoplasmas isolated from them. *J. comp. Path.* 88, 85-96.
- Jones, G.E., Buxton, D. and Harker, D.B. (1979). Respiratory infections in housed sheep, with particular reference to mycoplasmas. *Vet. Microbiol.* 4, 47-49.
- Jones, G.E., Field, A.C., Gilmour, J.S., Rae, A.G., Nettleton, P.F. and McLauchlan, M. (1982). Effects of experimental chronic pneumonia on bodyweight, feed intake and carcass composition of lambs. *Vet. Record* (in press).
- Nicolet, J. and Paroz, P. (1980). Tween 20 soluble proteins of Mycoplasma hyopneumoniae as antigen for an enzyme-linked immunosorbent assay. *Res. vet. Sci.* 29, 305-309.
- Stamp, J.T. and Nisbet, D.I. (1963). Pneumonia of sheep. *J. comp. Path.* 73, 319-328.
- Westphal, O., Luderitz, O. and Bister, F. (1952). Über die Extraction von Bakterien mit Phenol/Wasser. *Z. Naturforschung*, 76, 148-155.
- Wilkinson, S.G. (1977). Composition and structure of bacterial lipopolysaccharide. In "Surface Carbohydrates of the Prokaryotic Cell". (Ed. I.W. Sutherland, Academic Press London) 97-175.

**SPECIFICITY OF THE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR ANTIBODIES IN THE SERA OF SPECIFIC PATHOGEN-FREE LAMBS VACCINATED WITH *PASTEURELLA HAEMOLYTICA* ANTIGENS**

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**ABSTRACT**

Donachie, W., Burrells, C. and Dawson, A. McL., 1983. Specificity of the enzyme-linked immunosorbent assay (ELISA) for antibodies in the sera of specific pathogen-free lambs vaccinated with *Pasteurella haemolytica* antigens. *Vet. Microbiol.*, 8: 199–205.

Pooled serum from specific pathogen-free (SPF) lambs vaccinated with sodium salicylate extracted (SSE) antigens of *Pasteurella haemolytica* serotype A1 was shown to contain antibody to other A serotype SSE antigens when tested by the enzyme-linked immunosorbent assay (ELISA). Specific antibody to serotype A1 SSE antigens was demonstrated by absorption of the serum pool with heterologous serotype SSE antigens.

The type-specific antigens of serotypes A1 and A9 were prepared by phenol–water extraction (PWE) of their respective SSE antigens. The PWE antigens were examined in a sandwich ELISA where rabbit IgG anti-*P. haemolytica* A1 cells or A9 cells was used as a coating layer to bind PWE antigens. The specificity of these antigens was demonstrated by marked reduction of reactivity between serum from SPF lambs vaccinated with SSE of serotypes A1 or A9.

**INTRODUCTION**

Strains of *Pasteurella haemolytica*, the causative organism of ovine pasteurellosis, can be grouped into two biotypes (Smith, 1961) and 15 serotypes (Fraser et al., 1982a). Biotype T strains are associated with systemic pasteurellosis in older sheep while biotype A strains are responsible for septicaemia in young lambs and pneumonia in all ages of sheep. A recent survey by Fraser et al. (1982b) has shown that serotypes A1, A2, A6, A7 and A9 comprise 80% of the total number of biotype A strains submitted for examination.

The 15 serotypes of *Pasteurella haemolytica* are identified by an indirect haemagglutination (IHA) test (Biberstein et al., 1960). However, using the more sensitive indirect enzyme-linked immunosorbent assay (ELISA) for measurement of antibody to *P. haemolytica* (Burrells et al., 1979) the sera of mice and rabbits immunized with sodium salicylate extracted (SSE) anti-

gens of individual serotypes exhibited considerable cross reactivity (Burrells et al., 1983). In addition, sera from ewes and lambs injected with a trivalent *P. haemolytica* vaccine containing serotype A1, A2 and A6 SSE antigens when tested by ELISA reacted not only to vaccine components, but also to antigens of serotype A11 (N.J.L. Gilmour and C. Burrells, 19XX, unpublished observations). These observations suggest the existence within the A biotype of common group antigens not demonstrable by IHA.

This communication reports the demonstration of A1 serotype specificity by absorption of immune serum and the development of a modified ELISA for determining serum antibody to serotype-specific, phenol-water extracted antigens. The "sandwich" ELISA method described here used rabbit IgG anti-*P. haemolytica* serotypes A1 or A9 cells as a coating layer to bind type-specific antigens. This should allow the measurement by ELISA of the antibody response to individual serotypes of *P. haemolytica* in sheep vaccinated with multivalent *P. haemolytica* vaccines.

## MATERIALS AND METHODS

### *Antigens*

Sodium salicylate extracted (SSE) antigens of *P. haemolytica* were prepared as previously described (Wells et al., 1979a) and the bacterial cells remaining after sodium salicylate treatment were retained for use as absorbing antigen. Phenol-water extracted (PWE) antigens were prepared from A1 and A9 SSE by the hot phenol-water method (Westphal et al., 1952).

### *Antisera*

Aliquots of a previously prepared pool of specific pathogen-free (SPF) lamb anti-A1 serum (Wells et al., 1979b) were absorbed (Table I) at 37°C for 2 h then held at 4°C overnight. Resulting precipitates were removed by centrifugation at 1200 *g* for 20 min and the supernates retained for testing.

Individual antisera were obtained from 3 SPF lambs vaccinated with A1 SSE, using the method of Wells et al. (1979b), and from 3 lambs similarly vaccinated with A9 SSE. Control serum was obtained from 2 unvaccinated SPF lambs.

Hyperimmune rabbit sera against A1 and A9 cells, identified by IHA, were prepared by repeated injections of formalin-killed cells. Immunoglobulin G (IgG) fractions of these sera were prepared by fractionation on protein A-Sepharose CL-4B (Pharmacia (Great Britain) Ltd. Hounslow, Middlesex) (Goding, 1976) followed by dialysis against phosphate buffered saline (PBS).

TABLE I

Absorption of pooled antisera to *P. haemolytica* type A1 with heterologous SSE antigens and with homologous sodium salicylate treated cells

Serum aliquot	Volume of serum (ml)	Absorbant		PBS Final (ml) dilution
		SSE antigens	Sodium salicylate treated A1 cells (ml)	
1	0.2	—	—	2.8 1/15
2	0.2	0.2 ml each of A2, T3, T4, A5, A6, A7, A8, A9, T10, A11 and A12	—	0.6 1/15
3	0.2	—	0.2	2.6 1/15
4	0.2	0.2 ml each of A2, T3, T4, A5, A6, A7, A8, A9, T10, A11 and A12	0.2	0.4 1/15



### *Enzyme-linked immunosorbent assays (ELISA)*

Absorbed SPF lamb antiserum to type A1 was examined by an indirect ELISA technique as previously described (Burrells et al., 1979) with the exception that optical densities were determined using a "Titertek Multiskan" multichannel spectrophotometer (Flow Laboratories, Irvine, Ayrshire). Doubling dilutions of the absorbed serum in diluent (PBS/Tween 20) were added in 300- $\mu$ l volumes to triplicate wells of 96-well microtitre plates (Gibco Biocult, Paisley, Renfrewshire), previously sensitised with *P. haemolytica* A1 SSE, and also to wells similarly sensitised with another biotype SSE (A11). Results were recorded as optical densities at a wavelength of 405 nm ( $OD_{405}$ ).

PWE antigens were examined with SPF lamb sera in a "sandwich" ELISA based on that described by Burrells et al. (1979) as PWE antigens did not bind directly to microtitre plate wells.

The test consisted of six steps with three PBS/Tween 20 washes between each of the first five (300  $\mu$ l volumes of reagents at previously determined optimum dilutions were used). (1) Microtitre wells were coated with the IgG fraction of the rabbit antisera diluted in 0.05 M carbonate/bicarbonate buffer pH 9.6, by incubating at 4°C for 18 h. (2) A1 and A9 PWE (4.0  $\mu$ g ml<sup>-1</sup>) were added to wells precoated with homologous IgG antibody and incubated for 1 h at 37°C. (3) SPF lamb sera, diluted in PBS/Tween 20 were then added to appropriate wells and allowed to stand for 3 h at room temperature (RT). (4) Alkaline phosphatase conjugated pig IgG anti-sheep IgG (Burrells et al., 1979) diluted in PBS/Tween 20 was added to every well and allowed to stand at RT for 3 h. (5) Enzyme substrate (*p*-nitrophenyl phosphate, (Sigma Chemical Co., Poole, Dorset.) 1 mg ml<sup>-1</sup> in 10% diethanolamine buffer) was added to every well and left at RT for 1 h. (6) Enzymic degradation was stopped by adding 50  $\mu$ l of 2 M NaOH to each well and the  $OD_{405}$  for each was recorded.

### RESULTS

The various absorption procedures performed on SPF antiserum to type A1 are shown in Table I. The  $OD_{405}$  of each dilution of unabsorbed serum in reaction with A1 SSE and A11 SSE are given in Figs. 1 and 2, respectively. Although A1 specificity might be demonstrated by the difference in  $OD_{405}$  at dilutions beyond 1/240 (e.g., the  $OD_{405}$  at 1/480 with A1 SSE = 2.1, whilst with A11 SSE the  $OD_{405}$  = 1.1), cross-reaction with A11 SSE up to a dilution of 1/7680 was detected. Absorption with A1 cells which had been extracted with sodium salicylate did not reduce the  $OD_{405}$  at any point, and in the case of A1 SSE, slightly but consistently enhanced the reaction (Fig. 1).

When the SPF anti-A1 serum was absorbed with the SSE antigens of all the available serotypes other than the homologous SSE, either with or with-



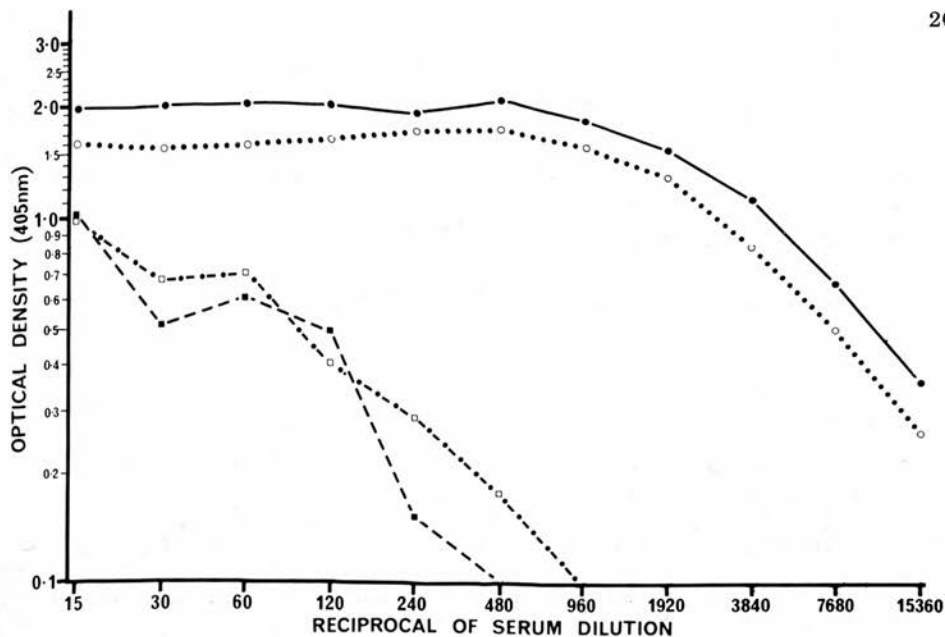


Fig. 1. Optical densities at 405 nm ( $OD_{405}$ ) of dilutions of SPF anti-A1 serum after ELISA reactions with A1 SSE antigen of *P. haemolytica*. Replicate aliquots of serum were unabsorbed (○), absorbed with sodium salicylate treated A1 cells (●) absorbed with SSE of other serotypes (■) or absorbed with SSE antigens plus sodium salicylate treated A1 cells (□).

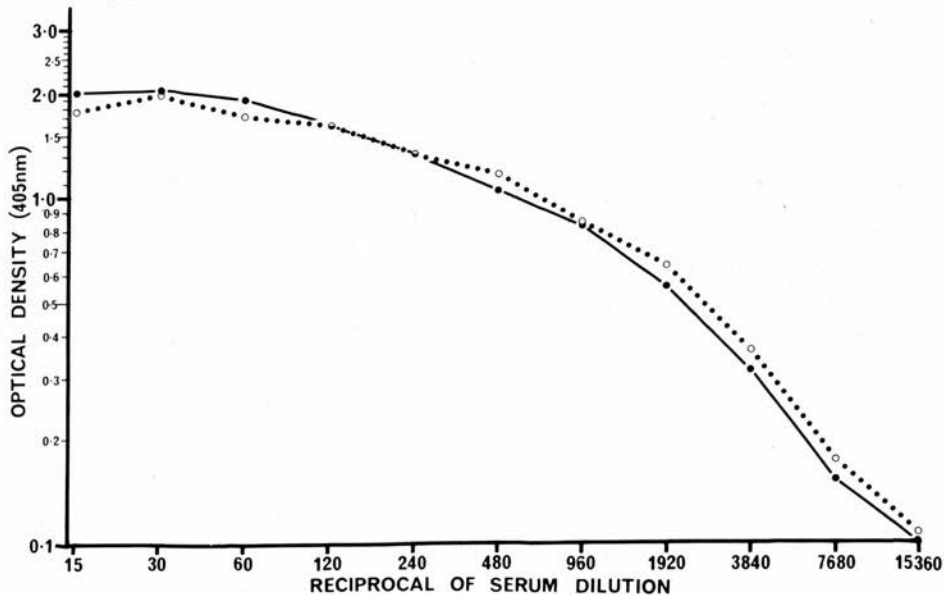


Fig. 2. Optical densities at 405 nm ( $OD_{405}$ ) of dilutions of SPF anti-A1 serum after ELISA reactions with A11 SSE antigen of *P. haemolytica*. Replicate aliquots of serum were unabsorbed (○) or absorbed with sodium salicylate treated A1 cells (●). (No  $OD_{405}$  was obtained when serum was absorbed with SSEs of other serotypes or SSE antigens + sodium salicylate treated A1 cells).



out SSE treated A1 cells, a marked reduction of OD<sub>405</sub> was observed with the homologous antigen (Fig. 1).

Type specificity for A1 was indicated by the fact that at all dilutions the same absorbed serum gave negative OD<sub>405</sub> values with A11 antigen.

Table II shows the results obtained when SPF lamb sera to A1 and A9 were tested against homologous and heterologous PWE antigens. Individual homologous values were all greater than 0.69 whilst heterologous values were less than 0.23. Paired sera from the two unvaccinated lambs remained negative to A1 PWE, but showed some reaction to A9 PWE. This reaction was also seen in the pre-vaccination sera of vaccinated lambs.

TABLE II

OD<sub>405</sub> values obtained from the reaction of antisera to type A1 or A9 with homologous and heterologous phenol-water extracted antigens

SPF lamb no.	Vaccine	A1 PWE		A9 PWE	
		Pre-vaccination	Post-vaccination	Pre-vacc.	Post-vacc.
1	A1 SSE	0	1.065	0.097	0.122
2		0	1.087	0.132	0.225
3		0	0.691	0.126	0.154
4	A9 SSE	0	0.025	0.096	1.140
5		0	0	0.117	0.865
6		0	0.030	0.084	0.696
7	Not vaccinated	0	0	0.086	0.151
8		0	0	0.117	0.130

## DISCUSSION

Pooled sera from SPF lambs immunised with *P. haemolytica* serotype A1 SSE were shown to cross-react with SSE of serotype A11. Absorption with pooled SSE antigens from all the other available serotypes left antibody which reacted specifically with type A1 SSE. The observed cross-reactivity has also been described in mouse and rabbit serum (Burrells et al., 1983). It is interesting that sodium salicylate-treated A1 cells failed to reduce the OD<sub>405</sub> when used to absorb antiserum to A1 SSE. This indicates that little, if any, of the antigens extracted by sodium salicylate remain on the cells after treatment.

PWE antigens of *P. haemolytica* SSE contain serotype-specific antigens which are detectable by the IHA test and are polysaccharide in composition (W. Donachie, 1980, unpublished observations). Antibodies to PWE antigens of serotypes A1 and A9 were measured by the modified ELISA. Although some pre-vaccination background reaction to A9 PWE, but not A1 PWE, was present in sera from both vaccinated and control lambs the lowest

OD<sub>405</sub> in a homologous system (lamb 3 serum = 0.691) was more than three times greater than the highest heterologous OD<sub>405</sub> (lamb 2 serum = 0.225). This was the criterion of specificity used by Russell et al. (1980) to measure antibody to pneumococcal polysaccharides. A "sandwich" ELISA was used, as coating layers of antiserum or IgG have previously been shown to bind pneumococcal polysaccharides efficiently and with reproducible results (Russell et al., 1980; Barret et al., 1980).

On the basis of these results with two *P. haemolytica* serotypes it is possible to determine the level of antibody to a single serotype. Further work is being carried out to test the applicability of this "sandwich" ELISA to the other serotypes as the specificity would allow quantitative assessment of the antibody response to individual components in multivalent *P. haemolytica* vaccines.

## REFERENCES

- Barret, J.D., Ammann, A.J., Stenmark, S. and Wara, D.W., 1980. Immunoglobulin G and M antibodies to pneumococcal polysaccharide detected by enzyme-linked immunosorbent assay. *Infect. Immun.*, 27: 411-417.
- Biberstein, E.L., Gills, M. and Knight, H., 1960. Serological types of *Pasteurella haemolytica*. *Cornell. Vet.*, 50: 223-300.
- Burrells, C., Evans, H.B. and Dawson, A. McL., 1983. Antigenic relationships between the serotypes of *Pasteurella haemolytica* demonstrable by enzyme-linked immunosorbent assay (ELISA). *Vet. Microbiol.*, 8: 187-198.
- Burrells, C., Wells, P.W. and Dawson, A. McL., 1979. The quantitative estimation of antibody to *Pasteurella haemolytica* in sheep sera using a micro enzyme-linked immunosorbent assay (ELISA). *Vet. Microbiol.*, 3: 291-301.
- Fraser, J., Laird, S. and Gilmour, N.J.L., 1982a. A new serotype (biotype T) of *Pasteurella haemolytica*. *Res. Vet. Sci.*, 32: 127-128.
- Fraser, J., Gilmour, N.J.L., Laird, S. and Donachie, W., 1982b. Prevalence of *Pasteurella haemolytica* serotypes isolated from ovine pasteurellosis in Britain. *Vet. Rec.* 110: 560-561.
- Goding, J.W., 1976. Conjugation of antibodies with fluorochromes: modifications to the standard methods. *J. Immunol. Methods.*, 13: 215-226.
- Russell, H., Edward, R.L., Wortham, E.W. and Facklam, R.R., 1980. Enzyme-linked immunosorbent assay for detection of antibodies against *Streptococcus pneumoniae* capsular polysaccharides. *J. Clin. Microbiol.*, 11: 198-199.
- Smith, G.R., 1961. The characteristics of two types of *Pasteurella haemolytica* associated with different pathological conditions in sheep. *J. Pathol. Bacteriol.*, 81: 431-440.
- Wells, P.W., Gilmour, N.J.L., Burrells, C. and Thompson, D.A., 1979a. A serological comparison of *Pasteurella haemolytica* vaccines containing different adjuvants. *Res. Vet. Sci.*, 27: 248-250.
- Wells, P.W., Evans, H.B., Burrells, C., Sharp, J.M., Gilmour, N.J.L., Thompson, D.A. and Rushton, B., 1979b. Inability of passively-acquired antibody to protect lambs against experimental pasteurellosis. *Infect. Immun.*, 26: 25-29.
- Westphal, O., Luderitz, O. and Bister, F., 1952. Über die Extraction von Bakterien mit Phenol/Wasser. *Z. Naturforsch.*, 76: 144-155.